Cross-Communication Between *Trichoderma* and Plants During Root Colonisation

---

A thesis submission in partial fulfilment for the requirements of the Degree of Doctor of Philosophy (Biochemistry) at Lincoln University.

---

at

The Bio-Protection Research Centre, Lincoln University.

by

Robert Lawry

January 2016

Abstract of a Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Ph.D. in Biochemistry

Cross-Communication Between *Trichoderma* and Plants During Root Colonisation

By Robert Lawry

Fungi of the genus *Trichoderma* are found in soils worldwide and have a range of lifestyles that bring them into interaction with agriculturally relevant plants. *Trichoderma virens* is a potential endophyte, and may be suitable for application as a bio-control agent due to its mycoparasitic ability. However, little is understood about the nature of *T. virens'* interactions with plant hosts. In this study, using *Zea mays* as an agriculturally relevant plant model, the nature of the interaction between *Trichoderma* and its plant hosts was examined. A novel interaction system, in sterile soil was established and maize hybrid line was determined to significantly affect *T. virens* colonisation. Microscopic analysis was used to visualise the ability of *T. virens* to penetrate, colonise and persist in the host plant. Appressoria-like structures were observed, and intra- and inter-cellular growth patterns were identified. Genomic and transcriptomic analyses were used to screen for potential effector proteins and other bioactive molecules involved in plant-fungal communication. *T. virens* communication with its host plant appears to be more reliant on lytic enzymes, polysaccharides and secondary metabolites than previously suspected, potentially reflecting its genetic origins as a saprobe. The expression of a large number of lytic enzymes by *T. virens* suggests that damage associated molecular pattern pathways would act as the primary elicitors of a plant response in maize during *T. virens* colonisation. Attempts to verify the role of lytic enzymes using an XlnR1 deletion mutant proved difficult due to ectopic recombination of deletion constructs; however the process yielded an enhanced protoplasting method for *T. virens* that was subsequently adopted by the wider research group. In summary, this work identified novel information on the mechanisms used by *T. virens* to successfully colonise host plants during an endophytic interaction.

Keywords: *Trichoderma virens*, endophyte, root colonisation, lytic enzymes, CWDE, DAMPs, plant-fungal communication.

Acknowledgements

The completion of this thesis has been a long journey, and the support of many people has ensured that I made it through all of the challenges I faced to reach the end. There are those whose input made a huge difference to both my quality of life and quality as a scientist during this study. I would like to particularly thank my PhD supervisors, Dr. Artemio Mendoza-Mendoza and Dr. Andrew (Andy) Pitman. Artemio has been a fantastic teacher of fungal molecular biology, and his enthusiasm for the work helped me persevere with the project through a lot of challenges. Andy's honest and insightful criticism has been absolutely invaluable in improving my skills as a scientific writer and as a critical thinker. I would also like to thank Professor Leo Condron for coming on-board with the project late and providing excellent advice, and Dr. Chris Brown, for taking the time to teach me RNA sequencing and transcriptome analysis. I would also like to thank Dr. David Greenwood for his advice on protein science and some exciting experimental techniques. I am also very grateful for the many small things done by the support staff at Lincoln and the Bio-Protection Research Centre, their hard work behind the scenes makes the life of a Ph.D. student much easier. I would like to thank the Marsden Fund and the Bio-Protection Research Centre for their support of the project.

Many people contributed immensely to my time at Lincoln University. I would like to thank the residents of the 'man cave', a base of operations like no other, defended by walls of stored insects, and protected by desks covered so thickly in paraphernalia , none could possibly encroach upon our realm. Without the sense of humour and numerous discussions had with my fellow cave dwellers, I would be considerably less sane. The names of the mighty heroes of the cave are too powerful to be written here, but you all know who you are. I would also like to thank the inhabitants of lab 612, for the good times both in and out of science, as well as some particularly great pranks.

I am immensely grateful for the support of my dad, mum, brother and gran during the long process of writing this thesis. I am very thankful for all of my friends, for their patience and support over the last few years (and for listening to so much science talk). I wish to thank Vikki Smith for her great advice and sense of humour, which added a lot to this thesis and life in general (and falcons!). I am also grateful to Laura Nixon, for many epic times whether tramping, drinking or playing board-games, and for reminding me not to be a hermit while writing up. I would like to thank Guillermo (Memo) Nogueira Lopez, for his sense of humour and positive attitude which has been a real inspiration during the challenges of the last few years. Finally I am very grateful for my friends who were all very supportive and made a real effort to keep in touch, despite the often antisocial nature of writing a Ph.D. thesis.

Abbreviations and Terms

Abbreviation	Meaning
Δ	Deletion
Avr	Avirulence
BIC	Biotrophic Interfacial Complex
CAP	Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein
CWDE	Cell Wall Degrading Enzyme
d	Day
DAMP	Damage Associated Molecular Pattern
DNA	Deoxyribonucleic Acid
DPI	Days Post Inoculation
EIX	Ethylene-Inducing Xylanase
ETI	Effector Triggered Immunity
Gb	Gigabase
GlcNac	N-acetyl-D-glucosamine
Gy	Grays (radiation)
h	Hour
HPI	H Post Inoculation
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
JGI	Joint Genome Institute
kb	Kilobases
LRR	Leucine Rich Repeat
LysM	Lysin Motif
M	Molar
MAMP	Microbe Associated Molecular Pattern
MAPK	Mitogen Activated Protein Kinase
min	Minute
mM	Millimolar
MSB2	Multicopy Suppressor of Budding defect 2
MTI	Molecular pattern Triggered Immunity
MYB72	myb domain protein 72
NBS-LRR	Nucleotide Binding Site - Leucine Rich Repeat
NGS	Next Generation Sequencing
nm	Nanometer
NPR1	Natriuretic Peptide Receptor 1
PAMP	Pathogen Associated Molecular Pattern
PC2	Physical Containment 2
PDA	Potato Dextrose Agar
PR	Pathogenesis Related
TSM	*Trichoderma* Selective Media

Table of Contents

Table of Figures

Table of Tables

1 Introduction

1.1 Plant Immunity, Endophytes and Key Definitions

The plant defence system is composed of a robust, three-layer mechanism consisting of one physical layer and two layers based on molecular pattern recognition that have significant crossover with each other. This system is capable of suppressing the majority of microbial invaders with only extremely well-adapted pathogens capable of causing sustained infections. The first layer of plant defence consists of a physical barrier that microbes must penetrate. The second layer consists of enzymes and receptors which trigger active immune responses. The third layer is composed of effector-detecting receptors, and is responsible for effector triggered immunity (ETI) (Jones & Dangl, 2006). Both the second and the third layer are capable of greatly escalating a plant's response to microbes, eventually triggering systemic immunity.

The first layer of plant immunity consists of barriers made from strong molecular bonds and crosslinking of complex polysaccharide and lipid molecules (Malinovsky, Fangel, & Willats, 2014). This layer is readily observable, consisting of wax, cuticle layers, cell walls and stomata, all of which must be overcome to gain physical entry into the plant (Bellincampi, Cervone, & Lionetti, 2014). This layer is discussed in greater detail in **Section 1.3**.

The second layer, that of microbe associated molecular pattern (MAMP) recognition, is more complicated. All microbes, whether beneficial or pathogenic, contain MAMPs. Pathogen associated molecular patterns (PAMP) consist of features common to only pathogens, but not microbes in general, such as endotoxins[1] (Thonart, Ongena, & Henry, 2012). A possible third component of the second immune layer is damage associated molecular pattern (DAMP) recognition. DAMP recognition allows the plant to recognise damage caused to itself by microbial invaders or colonisers (generally via lytic enzyme release products such as polysaccharide molecules) (Benedetti

[1] It is important to note that the definition of MAMPs and PAMPs in the literature is rather blurred. They have been used interchangeably and also to refer to separate concepts. For the purposes of this thesis MAMPs will refer to patterns common to microbes (e.g. flagellin, chitin) and PAMPs will refer specifically to those related to pathogens.

et al., 2015). MAMP, PAMP and DAMP recognition form the innate (or basal) plant immune system, now known as MTI (Molecular pattern triggered immunity) (Muthamilarasan & Prasad, 2013).

The third layer (ETI) is similarly complex. This layer is capable of causing severe and lasting responses to pathogens and is triggered in response to effector molecules (**see Section 1.5**). Effectors are produced by pathogens to suppress the MTI layer (Jones & Dangl, 2006; Muthamilarasan & Prasad, 2013). Effector molecules are part of a complex evolutionary arms race, and have previously been called Avr proteins, as microbes producing them were avirulent on plant hosts with the appropriate ETI receptors (G. B. Martin, Bogdanove, & Sessa, 2003). Proteins that trigger a response to effector molecules are known as R proteins, and generally contain a nucleotide-binding site (NBS) leucine-rich repeat domain (LRR) (McHale, Tan, Koehl, & Michelmore, 2006). ETI leads to downstream signalling and, if persistent, triggers accumulation of molecules such as salicylic acid which leads to Systemic Acquired Resistance (SAR) (Gao, Zhu, Kachroo, & Kachroo, 2015; Halim, Vess, Scheel, & Rosahl, 2006). This is the most potent and long lasting plant immune response, analogous to acquired immunity in humans.

Endophytic mutualistic fungi differ from pathogens in that they must maintain a stable relationship with the host. It has recently been shown that they too elicit an immune response whilst attempting to colonise their host even though they are generally beneficial (Van Wees, Van der Ent, & Pieterse, 2008). The immune response encountered appears to share significant similarity to the plant's response to pathogenic attack. Up to 40% of genes responding to mutualistic fungi may also be involved in defence responses (Van Wees et al., 2008). This suggests that pathogenic models may provide useful and important insights into the mechanisms by which mutualistic endophytes colonise their plant hosts.

1.2 The Rhizosphere and Plant Outer Cell Wall

Before a fungus can enter into a plant, it must first cross the rhizosphere and make contact with plant cell wall structures. The rhizosphere is a region of soil that is defined by the area associated with a plant root, comprised of unique micro-organisms that are influenced by plant root secretions (Hartmann, Rothballer, & Schmid, 2007). This region is rich in micro-organisms and represents a distinct environment from the bulk soil around it (Bakker, Berendsen, Doornbos, Wintermans, & Pieterse, 2013). The microbial community present in bulk soil determines the microbial structure in

the rhizosphere; however the plant can manipulate rhizosphere conditions to select for various beneficial micro-organisms, and this appears to play a role in suppression of plant pathogenic microbes (Bakker et al., 2013). For *Trichoderma* to colonise the root as an endophyte, it must first be able to pass through the rhizosphere, and survive long enough for root entry. Rhizosphere competence has been shown in many *Trichoderma* spp. and appears to influence endophytic ability of various strains (Cripps-Guazzone, 2014).

Once the plant is within the rhizosphere, it must contend with the outer cell wall structure and then penetrate the cell wall itself. The outer root cell wall is coverd with root mucilage, which has wide ranging effects on rhizosphere interactions and plant nutrient acquisition and also produces exudates that may influence the surrounding regions (Osborn, Lochey, Mosley, & Read, 1999; Traoré, Groleau-Renaud, Plantureux, Tubeileh, & Boeuf-Tremblay, 2000). Root mucilage in maize consists of polysaccharides, primarily composed of glucose, mannose, fucose, xylose and arabinose, with fucose appearing to make up about 20% of the structure (Bacic, Moody, & Clarke, 1986; Osborn et al., 1999). This may create another barrier to *Trichoderma* entry, which due to its high sugar content, may induce sugar degrading enzyme production.

1.3 Physical Barriers to Microbe Colonisation

For a microbe to invade a plant the physical barriers of the plant must be breached. The plant cell wall is composed of a complex interwoven linkage of cellulose, hemicellulose, pectin and lignin, all of which are arranged to maximise crosslinking (Malinovsky et al., 2014; Ochoa-Villarreal, Aispuro-Hernández, & Martínez-Téllez, 2012). This makes molecular attack upon the cell wall difficult – yet microbes have found a number of ways to do so. Pathogens may evade the cell wall and cuticle entirely by attempting to enter through stomata, as they provide a ready-made entrance to the plant – albeit one protected by guard cells (Arnaud & Hwang, 2015). Guard cells may be overcome by a pathogen's ability to initiate stomatal opening (Schulze-Lefert & Robatzek, 2006). Other fungi produce infection structures (appressoria or appressoria-like structures) which adhere to the cell wall. These resemble a hemisphere, with a flattened bottom in contact with the plant cell wall. On the underside of the appressorium a penetration peg forms, which is then mechanically forced through the plant cell wall. The melanised appressoria can exert sufficient force to breach membranes made of metals such as gold – allowing the fungus easy access to plant cells by penetrating through outer plant root and leaf surfaces (Deising, Werner, & Wernitz, 2000). Non-

melanised appressoria often lack the ability to penetrate surfaces. Arbuscular mycorrhizal fungi use similar structures, called hyphopodia to attach and facilitate entry on plant epidermal cells (Rich, Schorderet, & Reinhardt, 2014). Appressoria formation has been observed in a variety of fungi, but has yet to be confirmed as a mechanism of penetration in *Trichoderma* species. Other mechanisms of physical penetration include access through wounds on the plant. Fungi also use compounds or molecules such as cell wall degrading enzymes (CWDE) and cell wall loosening molecules (e.g. expansins such as swollenin), to penetrate the outer layers of the plant, and possibly derive nutrition in the process (Brotman, Briff, Viterbo, & Chet, 2008; Göhre & Robatzek, 2008) (CWDEs are discussed in greater detail in **Chapter 4** of this thesis). In brief, CWDEs target cell wall molecules for degradation, and a complex cocktail of enzymes may allow weakening or destruction of the plant's cell wall matrix. This may facilitate mechanical entry, or be a reliable method of penetration on its own.

1.4 MAMP and PAMP Triggered Interactions

Once within the intercellular spaces of the plant (the apoplast), a more complex series of interactions occurs. Major families of pathogens carry distinct molecular markers, several of which are critical to their survival. Examples of these include chitin or flagellin, both critical cell structure components of fungi and bacteria, respectively (Hückelhoven, 2007). The plant innate MTI system reacts to MAMPs allowing broad spectrum identification of non-plant tissue. MAMP receptors (ligand-binding receptor kinases) are capable of detecting elicitors and instigating an immune response proportional to the level of MAMPs detected (Bent & Mackey, 2007). These receptors are known as pattern recognition receptors (PRRs), consisting of a pattern-binding domain and a kinase domain allowing downstream signalling (Muthamilarasan & Prasad, 2013). The specific binding of flagellin (a MAMP) to FLS2 protein, an LRR receptor kinase (a type of PRR), is a good example of this (Gómez-Gómez & Boller, 2000). Upon receiving the flagellin-triggered signal from the PRR, several molecules (such as serine and cysteine proteases) are released, causing the breakdown of the MAMPs into component molecules, which are then re-detected by secondary signalling molecules, escalating the immune response (Göhre & Robatzek, 2008). A similar response is initiated when damage to the plant is detected via DAMPs. PRR receptors will in this case detect breakdown products of the plant itself, caused by pathogenic attack (Krol et al., 2010). These primary responses are predominantly characterised by localised reactions to the area under attack, consisting of

oxidative bursts, localised necrosis and release of proteases. The extent of signalling will determine whether or not the response becomes systemic. The process is tightly regulated as it has significant energy costs to the plant.

Microbes that are adapted to a particular host plant have a counter for their host's MAMP defence system. This counter takes the form of small secreted molecules, known as effectors. Effectors are an incredibly diverse set of proteins few of which are fully understood. Known effector mechanisms include scavenging of MAMPs, degradation of host proteases, and deregulation of primary and secondary signalling pathways in their host (Presti et al., 2015). This type of interaction tends to lead towards evolutionary arms races, with strong selection pressures for the pathogen to evade host immunity, and for the plant to retain it. A more detailed discussion of these interactions is found in **Chapter 2**.

1.5 Effector Molecules and Effector Triggered Immunity

Effector molecules used by pathogens to overcome MAMP-triggered immunity can also be their downfall as they may be detected by receptors responsible for ETI. Effector molecules produced by pathogens suppress plant MTI. Detection of effector molecules initiates an immune response that can lead to SAR. Pathogens release another suite of effectors which attempt to counter ETI; however these may be also detected by plant ETI. Thus ETI is the key determinant in the ability of plants to resist colonisation by pathogens (and other colonisers such as endophytes), and the effector suite of a pathogen is the major determinant of its ability to colonise the plant. As a consequence these effector molecules face significant evolutionary pressure.

Effector molecules can be difficult to identify, as they have very little homology to most known proteins. So far, the only common sequence element seen in many, but not all, fungal effectors is the high number of cysteine residues, which has possibly evolved as a defence against plant proteases (Stergiopoulos & de Wit, 2009). Recent evidence suggests that conservation of structure may allow better definition of effector groups (de Guillen et al., 2015). More is known of bacterial effectors, particularly those involved in the type III secretion system, which can now be identified via predictive tools (Arnold et al., 2009; Coburn, Sekirov, & Finlay, 2007). Tandem repeats have been major predictors of effector proteins in *Melampsora* spp., yet were entirely absent from other plant colonising fungi (Mesarich, Bowen, Hamiaux, & Templeton, 2015). The majority of known

effectors are secreted, and some are then translocated into the host cell (Catanzariti, Dodds, & Ellis, 2007). Despite the need for transport into the cell, only one putative translocation motif has been found in oomycetes, which is the RxLR motif, however its exact mechanism remains controversial (Birch et al., 2009; Jeffrey G Ellis, 2011; Petre & Kamoun, 2014). This motif has not yet been seen in fungal effectors. It is also possible for other forms of translocation to occur. The type three secretion system of bacteria directly injects effectors into the host cell. Other forms of effector may not need to enter the cell to be effective and recently the Lysin motif (LysM), thought to be responsible for scavenging chitin that is released from the fungus by plant chitinases, was examined in detail (de Jonge & Thomma, 2009; Sánchez-Vallet, Mesters, & Thomma, 2015). This motif is found in many fungi and may allow them to evade detection by the host by reducing the extent of the immune response elicited by chitin, essentially evading the chitin recognition PRR involved in MTI. Molecules with this motif have also been shown to be capable of eliciting an immune response in *Arabidopsis*, which indicates a possible ETI response to this effector (de Jonge & Thomma, 2009). This motif has been found in mutualistic fungi and may be part of the communication system established with their hosts.

Effector genes are found in the majority of pathogens, fungal pathogens with known effectors include but are not limited to *Cladosporium fulvum*, *Fusarium oxysporum*, *Magnaporthe oryzae*, *Phytopthora* spp. and *Ustilago maydis* (De Wit, Mehrabi, Van Den Burg, & Stergiopoulos, 2009; Presti et al., 2015). The vast majority of genes code for proteins shorter than 200 amino acids long, with between one and eight cysteine residues (Catanzariti et al., 2007). A number of these proteins were shown to be secreted, and to directly interact with a host R protein. However, several effectors may act in concert upon a single plant protein, making it difficult to fully determine individual contributions to pathogenicity (Pritchard & Birch, 2011). Several species of pathogenic and mutualistic fungi form complex feeding structures within cells, known as haustoria. It has now been shown that haustoria are capable of delivering effectors into host cells in certain pathogens e.g. *M. oryzae* and *Melampsora lini* (Dodds et al., 2006; Presti et al., 2015). These findings are important as they indicate that the haustoria may be a key site for molecular communication in fungi.

Effector triggered immunity detects effector molecules by identifying modifications to plant signalling pathways or receptors, which in turn causes up-regulation of many immune-related

genes, and activates the hypersensitive response (Feechan et al., 2015). When resistance (R) proteins, which are usually nucleotide binding site – leucine rich repeat (NBS-LRR) proteins, bind to an effector molecule, they activate downstream responses, such as MAPK, volatile organic compound production and modification of plant hormone levels (Cui, Tsuda, & Parker, 2015; McHale et al., 2006). Sufficient levels of plant hormones, particularly salicylic acid (SA), then lead to enhanced and systemic plant resistance to the majority of pathogens (Halim et al., 2006).

Several possible models of ETI activation have been postulated; namely the receptor ligand model, the guard model, the decoy model and the bait and switch model (Cui et al., 2015; Stuart, Paquette, & Boyer, 2013). The receptor ligand model suggests that R proteins bind effectors and signal to trigger immunity (previously known as the gene-for gene model). The guard model suggests that R proteins detect modification to effector target PRRs which then causes them to signal in place of the original PRR protein. The decoy model involves proteins that are similar to effector targets, but have no real function in the cell other than to signal when they are targeted by effectors. Finally, the bait and switch model suggests that PRR proteins are bound by secondary proteins that protect them from the effector's action whilst preserving their signalling capacity upon the binding of the decoy to an effector (Cui et al., 2015; Stuart et al., 2013). Preservation of pathogenic ability can therefore only occur when a pathogen is capable of evading or suppressing all of the above mechanisms of MTI and ETI.

The sequence of events, and evolutionary significance, of these interactions has for a long time been modelled using the Zig-Zag model, a highly influential concept that describes the timing and evolution of this process (**Figure 1**) (Jones & Dangl, 2006). The Zig Zag model implies four discrete stages of interaction: Firstly, PTI (or MTI) leading to a basal level of response. This is followed by the release of effectors, reducing the response to a level that is ineffective against the pathogen. MTI is then countered by ETI which restores and amplifies the initial response. This ETI may then be countered by further effectors, which may then be countered in turn by ETI. This model has recently faced criticism as it is proposed that the distinction between time scales may not be realistic in terms of direct molecular interactions (Pritchard & Birch, 2014). Correct identification of the timeframe of this process is critically important, as the timing of the interaction determines the rates of pathogen clearance or host death. It also suggests that the time period between the phases of MTI and ETI is very small. Finally, the evolutionary scale is important in a different context, but

for molecular work the organism's evolutionary capability is unlikely to change across the duration of a particular experiment.

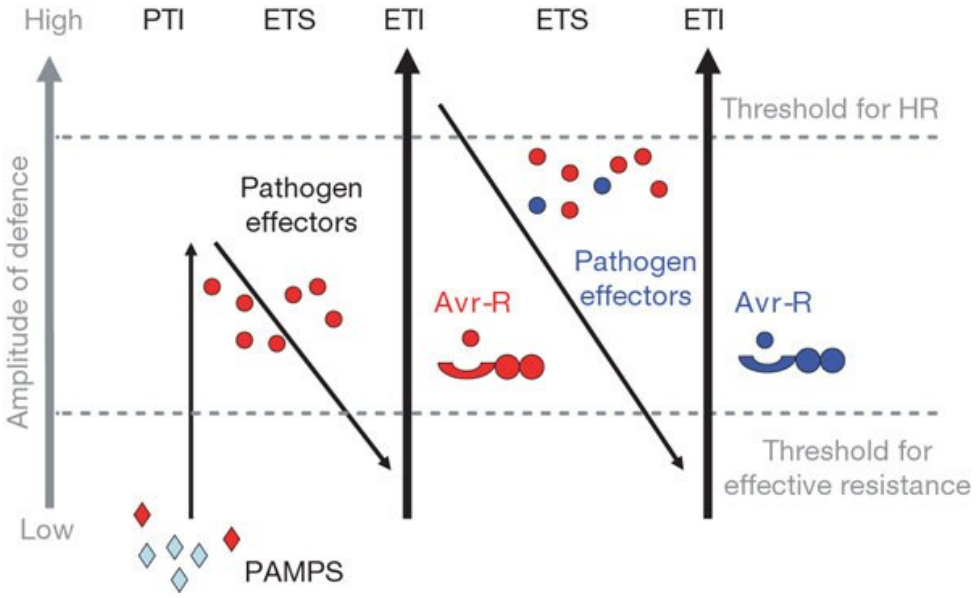


Figure 1: The zig-zag model of plant-pathogen interactions. This model was proposed by Jones and Dangl in 2006 (Jones & Dangl, 2006). It suggests four key time-points during an interaction, pathogen triggered immunity (PTI), effector triggered susceptibility (ETS), and effector triggered immunity (ETI). The last two (ETS and ETI) form an arms race, with the eventual winner successfully overcoming the other's defences.

1.6 R Proteins

Plant R proteins are a set of large, highly diverse proteins that are capable of detecting pathogen PAMPs or effector molecules. R proteins commonly contain a NBS and an LRR as well as a toll/interleukin-1 receptor or a coiled coil motif (De Wit et al., 2009; McHale et al., 2006). The LRR seems to be associated with protein specificity, whereas the NBS appears to function in signalling (McHale et al., 2006). NBS-LRR proteins can detect pathogens through direct or indirect interaction. Direct interactions involve the R protein interacting with the Avr protein in such a way as to disrupt its function, and have been observed *in vitro* using binding assays on proteins from *Magnaporthe oryzae* and its rice host (Catanzariti et al., 2007). These interactions form the ligand binding or gene-for-gene model of resistance. Indirect interactions occur when plant R proteins are capable of detecting modifications to other plant proteins, and have been observed in *Arabidopsis* RIN4 protein interactions with RPM1 and RPS2, which detect it being phosphorylated by pathogenic effectors (Mackey, Holt, Wiig, & Dangl, 2002). This system is efficient, as one protein can potentially guard against the effects of several types of effectors that act on the protein it is guarding; it may

also be more resistant to suppression by effectors than gene-for-gene systems (De Wit et al., 2009). Once a modification is detected, R proteins can activate signalling pathways to induce a number of defence responses, such as oxidative bursts, up-regulation of defence genes, and the hypersensitive response (McHale et al., 2006). These responses share many similarities to both PRR-based MTI and NBS-LRR based ETI, in that MAP kinases trigger further downstream responses, leading to hormonal changes throughout the plant. It is notable that deletion of proteins related to general disease susceptibility in *Arabidopsis* affected both ETI and MTI significantly, further supporting the crosslinking of these pathways. Furthermore, the evolutionary costs of maintaining large numbers of R proteins are thought to be high, potentially causing them to be maintained only under selection pressure (Tian, Traw, Chen, Kreitman, & Bergelson, 2003).

1.7 Endophytes, *Trichoderma* and Plant Colonisation

Endophytic mutualistic fungi differ from pathogens in that they must maintain a stable relationship with their host. Although these fungi are beneficial to the host, it has recently been shown that they also elicit an immune response, in a manner similar to that of pathogens (Presti et al., 2015; Van Wees et al., 2008). This immune response does not, however, escalate to the same levels as those involved in pathogen clearing. The plants response to mutualistic fungi is regulated by a complex communication system established between the plant and fungus. The exact mechanisms of this communication are unknown; however, signalling networks involving jasmonic acid, ethylene and salicylic acid are thought to be involved (Van Wees et al., 2008). These signalling networks are all part of systematic defence signalling pathways that are triggered in response to pathogenic attack (Shah, 2009).

Endophytes must manipulate plant signalling networks to adjust the plant's immune response. Plant defence proteins, such as NPR1 and MYB72, are involved in communicating with beneficial microbes and are also known to be part of defence signalling in response to pathogens (Rafiqi, Jelonek, Akum, Zhang, & Kogel, 2013; Van Wees et al., 2008). Several studies have shown that single gene alterations can seriously alter the interactions of both pathogens and endophytes; pathogenic species were rendered harmless, and endophytic species were made virulent, by addition or modification of single genes (Charlton, Shoji, Ghimire, Nakashima, & Craven, 2012; Eaton, Cox, & Scott, 2011; Kuo et al., 2014; H. Zhao et al., 2014). Particularly important is the ability of some fungi to switch lifestyle based on environmental or physiological conditions (Kuo et al.,

2014). It is clear that closely related pathways that respond to both pathogens and endophytes exist. These pathways are subject to extremely tight regulation. Understanding these will increase our knowledge of microbial communication, the interactions that determine pathogenicity, and may also aid us in understanding similar communication in human pathogens.

Trichoderma is a diverse genus of ascomycete fungi, with over 100 species currently known (Harman, Howell, Viterbo, Chet, & Lorito, 2004a). The genomes of three species, *Trichoderma atroviride*, *Trichoderma reesei* and *Trichoderma virens*, have been fully sequenced (http://genome.jgi-psf.org/). They have a wide variety of habitats and similarly diverse life histories. They are found in soils worldwide, but also function as opportunistic avirulent plant symbionts (Harman, Howell, Viterbo, Chet, & Lorito, 2004b). The ability of *Trichoderma* spp. to thrive in such a wide range of habitats is linked to their ability to produce a number of bioactive molecules, such as lytic enzymes and antibiotics (Woo, Scala, Ruocco, & Lorito, 2006). This has made them popular for use in the industrial biosynthesis of compounds such as cellulases, xylanases and chitinases (Felse & Panda, 1999; Mehrdad Azin, 2007). *Trichoderma* spp. are frequently found in symbiotic relationships with plants and are capable of increasing plant tolerance to a wide range of abiotic and biotic stresses. This makes them popular as biocontrol agents in agriculture (Kubicek et al., 2011). *Trichoderma* spp. are therefore good candidates for studies of host-microbe interactions.

Trichoderma spp. can function as symbiotic endophytes of several plant species, including maize and tomato (Morán-Diez et al., 2015; Sobowale, Cardwell, Odebode, Bandyopadhyay, & Jonathan, 2007). *Trichoderma* spp. penetrate plant root tissue and are assumed to use a penetration peg to punch through cell walls (Shoresh, Harman, & Mastouri, 2010). As the fungus enters the plant's roots, the plant deposits callose, limiting the extent of fungal growth (Ellinger et al., 2013; Luna et al., 2011). Interactions inside the plant cell are not well-studied, but several other pathogenic fungi and oomycetes form haustoria, which allow uptake of nutrients from the cell, as well as chemical signalling across the extra-haustorial membrane (Catanzariti et al., 2007; Jones & Dangl, 2006). Once settled within the plant, the fungus has a wide range of effects upon its host. Root growth, nutrient uptake and plant growth are all significantly enhanced by *Trichoderma* colonisation (Harman et al., 2004b). Perhaps most significantly, the plant's tolerance to pathogenic attack is increased. *Trichoderma* enhances the immune response predominantly by inducing the plant

immune system, but also by mycoparasitism and antibiosis (Shoresh et al., 2010). Maize is expected to derive some or all of these benefits from *Trichoderma* colonisation.

Plant defence is induced by *Trichoderma* through a number of methods. Recently Sm1 (and homologous proteins Epl1 and Sm2), a protein related to pathogenic phytotoxic proteins, was discovered to be secreted by both *T. atroviride* and *T. virens* (Djonovic et al., 2007; Frankie K. Crutcher, 2015; Gaderer et al., 2015; Salas-Marina et al., 2015). This protein is not toxic to the plant, and instead induced a systematic plant immune response. Deletion of the Epl1 gene in *T. atroviride* reduced the protective effect in tomato plants (Salas-Marina et al., 2015, p. 1). Terpenoids, peptabiols and other secondary metabolites synthesised by *Trichoderma* have also been shown to induce plant immune responses (F. Vinale, 2008; Shah, 2009; Shoresh et al., 2010). This results in a heightened response to pathogenic attack via priming of induced systemic resistance (ISR), and is less costly to the plant than constitutive expression of resistance genes. How *Trichoderma* evades the immune response itself is not yet clear and represents the main aim of this research.

Understanding the molecular dialogue between plants and mutualists is essential to the safe and effective use of biocontrol agents such as *Trichoderma* in agriculture. While these fungi are capable of increasing crop yields and protecting crops from biotic and abiotic stress factors, the lack of a clear explanation for the difference between pathogenic and endophytic lifestyles is troubling. Similar molecules seem to be involved in both pathogenic and mutualistic interactions. An in-depth study of *Trichoderma* in interaction with an agriculturally relevant plant will increase our understanding of the process of colonisation and the differences between endophytism and pathogenicity (Harman et al., 2004b). Pathogenic organisms have been well studied, however the focus with beneficial microbes has mainly been on their antibiotic properties. *Trichoderma* spp. are good candidates as they exist as mutualists with agriculturally relevant plants and are already in use in agriculture, making findings readily applicable.

1.8 Key Questions

It is apparent that data on plant-endophyte interactions is scarce in several key areas. Firstly, there is a limited understanding of the factors that influence host specificity and colonisation ability in *Trichoderma*. There is also little evidence for morphological structures that allow *T. virens* strain Gv 29.8 to enter plant hosts. The genetic components behind the interaction are also not well

understood. These topics are investigated in this thesis in the hope of determining the general genetic and physiological attributes that *T. virens* uses to act as a root endophyte of maize, and provide some ability to extrapolate this to other *Trichoderma* species, such as *T. atroviride* and *T. reesei*.

Chapter 3 asks three key questions: Firstly, does *T. virens* possess endophytic capability in maize? Secondly, what effect does maize host line have on fungal colonisation? And thirdly what morphological features enable entry into the host plant?

Chapter 4 examines the genetic components behind the interaction via *in silico* techniques. This section was guided by two key questions: do the *Trichoderma* species *T. atroviride, T. reesei* and *T. virens* have similar genetic components that resemble known effector genes? And what genetic characteristics exist in these *Trichoderma* spp. that may be relevant to plant colonisation? These questions aimed to identify general trends in *Trichoderma* spp. genetics and relate them to *T. virens* colonisation.

Chapter 5 aimed to determine which key genes or gene groups are differentially expressed during root-*T. virens* interaction, following the hypothesis that predicted small secreted effector proteins should be up-regulated during colonisation.

Chapter 6 took two highly differentially expressed candidate genes from the differential expression analysis in Chapter 5, and tested whether these genes had an effect on root colonisation *in vivo* with the aim of confirming their involvement in host colonisation. This should characterise the effect of these genes upon root colonisation.

2 General Materials and Methods

2.1 Materials, Kits and Sources

General kits (**Table 1)**, media and methods used in all subsequent chapters will be presented here. Methods relevant to specific chapters will be presented where appropriate.

Table 1: Kits used in this study and their purpose

Kit Name	Purpose
Ambion Turbo DNA-Free (Thermo-Fisher)	DNA-digestion
PCR-Select cDNA Subtraction Kit (Clontech)	cDNA Suppressive Hybridisation
Phusion High-Fidelity DNA Polymerase (NEB)	PCR (Polymerase Chain Reaction)
NEB Phusion Taq Polymerase (NEB)	PCR
Promega Wizard SV Gel (Promega)	Gel and PCR clean-up
pGEM-T (Promega)	Subcloning PCR products
Nucleospin Plasmid Mini (Macherey-Nagel)	Plasmid DNA extraction and purification
Nucleobond Midi (Macherey-Nagel)	Plasmid DNA extraction and purification
Nucleobond Maxi (Macherey-Nagel)	Plasmid DNA extraction and purification
Qiagen RNeasy (Qiagen)	RNA isolation and purification
TA-Cloning Kit pCR2.1 vector(Invitrogen)	pCR 2.1 vector and subcloning PCR products
Zymo Plasmid Miniprep	Plasmid DNA extraction and purification
Faststart Taq Polymerase (Sigma Aldrich)	PCR

2.2 Organisms and Strains

The following organisms and strains were used during the course of this Ph.D. (**Table 2**).

Table 2: Organisms and strains used for the experiments presented in this thesis.

Organism	Strain/Line	Source
Escherichia coli	Top10F	Invitrogen
Trichoderma atroviride	IMI206040	Dr. Alfredo Herrera-Estrella (Langebio, Mexico)
Trichoderma hamatum	FCC207	TrichoBank (Lincoln University)
Trichoderma harzianum	FCC261	TrichoBank (Lincoln University)
Trichoderma reesei	QM6a	ATCC 13631**
Trichoderma virens	Tv10.4	Professor Charles Kenerley (Texas A&M University, USA)
*Trichoderma virens**	Gv 29.8	Professor Charles Kenerley (Texas A&M University, USA)
Zea mays	34H31	Pioneer Seeds
Zea mays	33M54	Pioneer Seeds
Zea mays	34F95	Pioneer Seeds
Zea mays	34K77	Pioneer Seeds

Zea mays	35A30	Pioneer Seeds
Zea mays	36B08	Pioneer Seeds
Zea mays	36M28	Pioneer Seeds
Zea mays	37Y12	Pioneer Seeds
Zea mays	38V12	Pioneer Seeds
Zea mays	39G12	Pioneer Seeds
Zea mays	39T45	Pioneer Seeds
Zea mays	P0537	Pioneer Seeds
Zea mays	G30	Chieftain Seeds
Zea mays	F1	Chieftain Seeds
Zea mays	Popcorn	New World Supermarket

**T. virens* Gv29.8 was used for all experiments in this thesis except protoplasting. When *T. virens* is mentioned, it will be this strain unless otherwise specified. **American Type Culture Collection

2.3 Media

All media (**Table 3**) were made up to 1 L with dH_2O and sterilised by autoclaving at 121°C for at least 15 min unless otherwise specified. Media containing thermally unstable chemicals were filter sterilised by vacuum filtration through a 0.22 µm filter into a previously autoclaved container.

2.3.1 Bacterial Growth Media

Strains of *Escherichia coli* were propagated overnight at 37°C in either 5 mL LB broth in a 20 mL universal tube, or on LB + Agar plates containing 25 mL of LB Agar. Liquid cultures were shaken at 180 RPM. Selective media was prepared with the addition of 100 µg/mL ampicillin when required. IPTG (0.1 M final concentration) and X-gal (20 mg/mL) were added to solid selection media when required. Stock cultures were maintained at -80°C in a final concentration of 25% glycerol.

Table 3: Growth media for microorganisms and required supplements

Media	Components
LB Broth (Difco)	Tryptone 10 g/L Yeast Extract 5g /L NaCl 10 g/L
LB Agar (Difco)	Tryptone 10 g/L Yeast Extract 5g /L NaCl 10 g/L Agar 15 g/L
Glucose-Yeast Extract-Casein (GYEC)	Casein 20 g/L Yeast Extract 10 g/L

	Glucose 10 g/L
PDA (Difco)	Potato Starch Infusion 4 g/L Dextrose 20 g/L Agar 15 g/L
PDB (Difco)	Potato Starch Infusion 4 g/L Dextrose 20 g/L
Minimal Media	**Vogel's Salts** 20 mL Sucrose 15 g/L
Vogel's Salts	Sodium Citrate 150 g/L KH_2PO_4 250 g/L NH_4NO_3 100 g/L $M_gSO_4.7H_2O$ 10 g/L $CaCl_2.2H_2O$ 5 g/L **Trace elements** 5 mL **Biotin Solution** 2.5 mL
Trace Elements (100 mL)	Citric acid 5g $ZnSO_4.7H_2O$ 5g Fe $(NH_4)2$ $(SO_4)_2.6H_2O$ 1g $CuSO_4.5H_2O$ 0.25g $MnSO_4.H_2O$ 0.05g H_3BO_3 0.05g $Na_2MoO_4.2H^2O$ 0.05g
Biotin Solution (50 mL)	Biotin 5 mg
***Trichoderma* Selective Media (TSM)**	Agar 20 g/L Glucose 3 g/L Ammonium Nitrate 1 g/L $K_2HPO_4.3H_2O$ 0.9 g/L $MgSO_4.7H_2O$ 0.2g/L KCl 0.15 g/L Terrachlor 75 WP 0.2g/L Rose Bengal 0.15 g/L Chloramphenicol (2.5 mg/mL stock) 1 mL **Metal Salt Solution 1 mL**
Metal Salt Solution	$FeSO_4.7H_2O$ 1 g/L $MnSO_4.4H_2O$ 0.65 g/L $ZnSO_4.7H_2O$ 0.9 g/L

2.3.2 Fungal Growth Media

Fungal isolates were propagated on PDA, PDB, GYEC media or minimal media. Cultures were inoculated by placing 5 drops of spores on the plate or in the solution. Cultures were incubated at 25°C for 7 days. Liquid cultures (PDB, GYEC, Minimal media) were shaken at 180 RPM. Where required Hygromycin B was added at a final concentration of 200 µg/mL. Stock cultures were stored at -80°C in 50% glycerol at a 1:1 ratio with the culture solution.

2.3.3 Plant Growth Media

John Innes loam, potting mix and soil from monitored fields were used for plant growth (**Table 4**). All soils used for final experiments were gamma ray sterilised. Initially autoclaving was used but it was found to have imperfect sterilisation results (discussed in **Chapter 3**). Plants grown hydroponically were placed in 50 mL of an autoclaved water-Hoagland's basal salt mixture solution, with the seed supported by a plug of sterile cotton wool.

2.3.1 Protoplasting Media

Fungal digestion was performed in osmotic media (**Table 5**). Three different osmotic media were trialled in this study. Fungal digestion was performed with 0.24g cellulase and 0.5g Glucanex (Novozyme) per 50 mL of osmotic media.

2.3.1 Fungal Transformation Media

Fungal transformation was performed via polyethylene glycol (PEG) mediated DNA uptake. The PEG 3500 solution was prepared with 40g of PEG 3500 in 100 mL of 0.7 M Mannitol osmoticum.

Table 4: Growth media for plants

Media	Components
Hoagland's Basal Salt Mixture No. 2 (Sigma-Aldrich)	Ammonium phosphate 115.03 mg/L Boric acid 2.86 mg/L Calcium Nitrate 656.4 mg/L Cupric sullipide.5H2O 0.08 mg/L Ferric tartrate.2H2O 5.32mg/L Magnesium sullipide 240.76 mg/L Manganese chloride.4H2O 1.81 mg/L Molybdenum trioxide 0.016 mg/L Potassium Nitrate 606.6 mg/L Zinc Sullipide.2H2O 0.22 mg/L
John Innes Loam (Potting Mix)	7 kg Loam 3 kg Peat 2 kg Sand 0.6 kg Limestone (ground) 1.2 kg Hoof and Horn 1.2 kg Superphosphate 600 g Potassium Sulphate
Soil Analysis Results*	Phosphorus 102 mg/L Potassium 1.72 me/100g Calcium 14.3 me/100g Magnesium 4.53 me/100g Sodium 0.4 me/100g Nitrogen 77 kg/ha Mineralisable N 57 ug/g Carbon 6.2%

*Full results shown in supplementary material. The cation exchange capacity is measured by milliequivalents per 100g (me/100g).

Table 5: Protoplasting media

Media	Components
OM Media (pH 5.8)	1.2 M MgSO4.7H2O 100 mM NaH2PO4
Mannitol 0.6 M (pH 5.5)	50 mM CaCl2 0.6 M Mannitol 50 mM MES hydrate
Mannitol 0.7 M (pH 5.5)	50 mM CaCl2 0.7 M Mannitol 50 mM MES hydrate

2.4 Primers

Primer oligonucleotides were ordered and synthesised by Integrated DNA technology (IDT).

Table 6: Primers used during this study.

Primer Name	Sequence (5' -> 3')	Experiment
47927 OPL	GCCGAGTCAGCCACGAGAATAG	XlnR Deletion
47927OPR	CCTCGTGAATCCGCCTCTCA	XlnR Deletion
47927 LF L	AAAGGCAAGGCTCATGCCATTTAG	XlnR Deletion
47927 LF R SfiI	AACGGGCCATCTAGGCCAACGGGCCTTGTGGAAGAAGTC	XlnR Deletion
47927 RF L Sfi I	GGCCTGAGTGGCCTTGTACCGGTGGACAAAG	XlnR Deletion
47927 RF R	TTTCAATAGGTCGTCCATCAG	XlnR Deletion
47927 LFAcc65I	AAAGGTACCAGCCCTTTGTCGTGTTTC	XlnR Deletion
47927 RF XbaI	TTTTCTAGACAATAGGTCGTCCATCAG	XlnR Deletion
47927 Complementation Left Acc65I	AAAGGTACCGGCAAGGCTCATGCCATTTAG	XlnR Deletion
47927 Complementation Right XbaI	TTTTCTAGACCACCATCACTGATGCAAGTC	XlnR Deletion
58714 LF L	AAACGCCTTGAAACGGTATATCG	XlnR Deletion
58714 LF R Sfi I	AACGGGCCATCTAGGCCTGTCGGGATACGCAGAATAG	XlnR Deletion
58714 RF L Sfi I	GGCCTGAGTGGCCTCTTGCGTTGTACCGATG	XlnR Deletion
58714 RF R	TTTAGTCCCGTTTGAACTCTG	XlnR Deletion
58714 LFAcc65I	AAAGGTACCCGCCTTGAAACGGTATATCG	XlnR Deletion
58714 RF Xba I	TTTTCTAGAAGTCCCGTTTGAACTCTGTG	XlnR Deletion
58714 Complementation Left Acc65I	AAAGGTACCCGCCTTGAAACGGTATATCG	XlnR Deletion
58714 Complementation Right XbaI	TTTTCTAGAATTTGCTGCCAGCCATCTC	XlnR Deletion
58714 OPR	ACCACGGCAATAAACTGTCG	XlnR Deletion
58714 Complementation Left Acc65I	AAAGGTACCCGCCTTGAAACGGTATATCG	XlnR Deletion
58714 Complementation Right XbaI	TTTTCTAGAATTTGCTGCCAGCCATCTC	XlnR Deletion
M13 Forward Primer	TGTAAAACGACGGCCAGT	Sequencing PCR
M13 Reverse Primer	CAGGAAACAGCTATGAC	Sequencing PCR
oAM-LU 347	GGATGCCTCCGCTCGAAGTA	XlnR Deletion
oAM-LU 348	CGTTGCAAGACCTGCCTGAA	XlnR Deletion
oAM-LU 357	TTAGCGGCCGCCCGACGTTAACTGATATTGAAGGAGCA	HygR Not1 5'
oAM-LU 358	TTAGCGGCCGCCGTTAACGGAACCCGGTCGG	HygR Not1 3'
oAM-LU 359	TTAGGTACCATAACGGTGAGACTAGCGGCCGGT	Acc65I pki promoter
oAM-LU 360	TAACTCGAGGCGGTTAAGAGGGTTCTTCCGGCTTCGCGATAAGGTACT	XhoI pki Promoter
oAM-LU347	GGATGCCTCCGCTCGAAGTA	HY
oAM-LU348	CGTTGCAAGACCTGCCTGAA	YG

2.5 Vectors

Both vectors for *E. coli* transformation contained ampicillin resistance cassettes, and ampicillin was used as a selection antibiotic for all *E. coli* transformants. Fungal transformants were selected for using hygromycin resistance via an introduced resistance cassette. Cloning of products was verified by blue-white screening (indicating disruption of the *lac* operon by the insert – white colonies are selected), followed by polymerase chain reaction (PCR) and agarose gel electrophoresis verification of selected colonies. Final products were verified by sequencing using M13 primers.

2.5.1 Cloning Vectors for *E. coli*

pCR 2.1 was used for cloning PCR products. This vector contains ampicillin and kanamycin resistance cassettes and contains the *lac* operon for blue-white colony screening. Inserts were verified by sequencing using the M13 forward and M13 reverse primers. pCR2.1 is a 3.9 kb plasmid.

pGEMT vector was used for cloning PCR products. This vectors contains an ampicillin resistance cassette as well as the *lac* operon for blue-white colony screening. Insertions may also be verified by M13 primer sequencing.

2.5.2 Vectors Created for *T. virens* Transformation

pAMT-30 contains the phosophotransferase of hygromycin under the control of trp1 promoter (Mendoza-Mendoza, unpublished). This plasmid was used to create the following vectors.

pGEMT-47927LF-HY vector. A 1.1-Kb region of the 5' region of 47927 (Gene Identification number in the JGI *T. virens* V2 genome available at the Joint Genome Institute (JGI)) gene was amplified using *T. virens* Gv29.8 genomic DNA as template and primer combination 47927 LF R SfiI and 47927 OPL. The resulting PCR product was digested with *Sfi*I restriction enzyme and ligated to the 1.4-kb SfiI hygromycin resistance cassette fragment from PAMT-30 (Mendoza-Mendoza, unpublished). To generate the 47927LF-HY product, the ligation product was amplified with Phusion Taq polymerase using the primer combination 47927 LF L and oAM-LU347. The resulting PCR product was gel purified, A-tailed and ligated into plasmid pGEMT, generating plasmid pGEMT- 47927LF-HY. The resulting plasmid was amplified in Top10 F' *E. coli* and sequenced. pGEMT- 47927LF-HY was used for overlap transformation of *T. virens* Gv29.8 alongside pGEMT- 47927RF-YG.

pGEMT-47927RF-YG vector. A 1.1 Kb region 3' of the 47927 gene was amplified from *T. virens* genomic DNA using primers 47927 RF L Sfi I and 47927 OPR. The vector was created in the same

manner as pGEMT-47927LF-HY except the primer combination 47927 RF-R and oAM-LU348 was used. Plasmid pGEMT-47927RF-YG was used for overlap transformation alongside pGEMT-47927LF-HY.

pGEMT-58714LF-HY A 1.1 Kb region 5' of the 58714 gene was amplified from *T. virens* genomic DNA using primers 58714 LF R Sfi I and 58714 OPL. The vector was created in the same manner as pGEMT-47927LF-HY except the primer combination 58714 LF-L and oAM-LU347 was used. Plasmid pGEMT-58714LF-HY was used for overlap transformation alongside pGEMT-58714RF-YG.

pGEMT-58714RF-YG A 1.1 Kb region 3' of the 58714 gene was amplified from *T. virens* genomic DNA using primers 58714 RF L Sfi I and 58714 OPR. The vector was created in the same manner as pGEMT-47927LF-HY except the primer combination 58714 RF-R and oAM-LU348 was used. Plasmid pGEMT-58714RF-YG was used for overlap transformation alongside pGEMT-58714LF-HY.

2.6 General Methods

2.6.1 DNA Isolation

Phenol:Chloroform:Isoamylalcohol extraction was used to obtain genomic DNA from *Trichoderma* isolates (Sambrook & Russell, 2001). Approximately 100 mg of mycelial tissue from 1 day old PDB suspensions was harvested and ground under liquid nitrogen. Tissue was placed in a 2 ml tube with 0.5 mL of DNA extraction buffer (100 mM TRIS-HCl pH 7.5, 50 mM EDTA pH8, 1.5 M NaCl, 2% CTAB and 0.05% β-mercaptoethanol). A 0.5 mL mix of 25:24:1 phenol:chloroform:isoamylalchohol was added and then the tube was vortexed for 10 seconds. Following this, the sample was centrifuged for 10 min at 13,000 rpm. The top layer is then removed via careful pipetting and placed in a new tube, where 2.5 volumes of ice cold ethanol were added. The tube was then mixed by inversion before centrifuging again for 5 min at 13,000 rpm. The supernatant was then discarded and the centrifugation was repeated. Any remaining ethanol was removed and then the DNA pellet was re-suspended in 100 µL sterile H_2O. Resulting DNA was quantified by Nanodrop and verified by agarose gel electrophoresis.

In the event of RNA contamination, a 10 µL solution of 10 mg/mL of RNAse A was added and incubated for 1 h at 37°C. DNA was precipitated by addition of 0.1 volumes of Sodium Acetate (3 M, pH 5.5) with 2.5 volumes of 100% ethanol. The tube was mixed by inversion and chilled at -20°C for 1 h. The sample was then centrifuged for 10 min at 13000 RPM before removing the supernatant.

The pellet was then washed in 100 µL 70% ethanol before air drying and re-suspension in 100 µL sterile H_2O.

2.6.2 RNA Isolation from *T. virens* in Interaction with Maize

Total RNA isolation was carried out from approximately 200 mg of ground *T. virens*-inoculated maize root tissue taken from the 2 cm root section closest to the seed. The Qiagen RNeasy kit was used according to the kit's instructions, except that 700 µL of RLC buffer was used instead of 450 µL. RNA quantity was determined by Nanodrop analysis, and RNA quality was verified by denaturing agarose gel electrophoresis.

2.6.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to determine the quality of DNA and RNA extractions, and determine the successful amplification of PCR products via size separation. 0.8% agarose gels were prepared by addition of 3.2 g of agarose to 400 mL of 1x TAE. The solution was then heated until the agarose was fully dissolved. 1x TAE was prepared from a stock solution of 50 x TAE buffer (2 M Tris-HCl, 2 M acetic acid, 50 mM EDTA, pH 8). Agarose solution was cooled and 1 µL of Red-Safe was added per 100 mL of gel. Gels were then poured into a casting tray containing a single comb. DNA or RNA samples were then mixed with an appropriate loading dye and pipetted into the sample wells. The gel was run for 35 min at 110 V and 500 mA. Bands were then observed under UV light and digitalised using a Bio-Rad Molecular Imager VersaDoc MP 4000.

2.6.4 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was used to amplify inserts for cloning and to verify transformations. Phusion High-Fidelity DNA Polymerase (NEB) was used to ensure error-free cloning of inserts. Phusion PCR uses slightly elevated temperatures compared to most other commercial Taq polymerases. FastStart Taq DNA Polymerase (Sigma-Aldrich) was used for standard PCRs. The standard protocol for each is shown below:

Table 7: Standard PCR protocols for Faststart and Phusion Taq polymerases

Cycles	Step	Faststart Taq Polymerase (Sigma-Aldrich)		Phusion® High-Fidelity DNA Polymerase (NEB)	
		Temperature (°C)	Time	Temperature (°C)	Time
1X	Initial Denaturation	95	4 min	98	30 s
35X	Denaturation	95	30 s	98	5 - 10 s
	Annealing	55-65	30 s	50-65	10 - 30 s
	Elongation	72	90 s	72	15 - 30 s/kb
1X	Final Elongation	72	7 min	72	10 min

2.6.5 TA Cloning of Inserts

Both pGEMT and PCR2.1 vectors were capable of accepting inserts via TA ligation. PCR products containing a single 3´ adenine overhang were ligated (using T4 DNA ligase) into a linearised vector containing a complementary 3' thymine overhang. Two ligation procedures were used; time period for ligation was either 1 h or overnight, dependent on whether the 1 h ligation was initially successful.

Table 8: Ligation Protocol for T4 and Rapid Ligase

T4 Ligase
1 µL T4 Ligase
3 µL insert
1 µL vector
1 µL 10 x Ligation Buffer
4 µL dH_2O
pGEMT Rapid Ligase
5 µL Rapid Ligase Buffer (2x)
1 µL T4 Ligase
1 µL pGEM-T Vector
2 µL PCR product
1 µL dH_2O

2.6.6 Bacterial Transformation via Heat Shock

Transformation of *E. coli* was carried out by heat shock treatment of TOP10F' chemically competent cells. Firstly 10 µL of plasmid DNA was added to 40 µL of TOP10F' cells. The mixture was then placed on ice for 10 min prior to heat shock treatment at 42°C for 40 s. The mixture was then returned to

ice for 10 min and then added to 300 µL of SOC media at 37°C. This mixture was then incubated for 1 h at 37°C shaking at 200 RPM. Cells are then plated onto LB + Ampicillin + IPTG + X-gal plates and grown overnight at 37°C. White colonies were selected for further analysis.

2.6.7 Plant Growth Methods

Seed sterilisation and plant growth methods were determined by the investigations presented in **Chapter 3**. The methods presented here are the final methods and were used for all subsequent experiments.

2.6.7.1 Seed Sterilisation

Maize seeds were placed in a 50 mL tube containing 95% ethanol and stirred for 7 min. Seeds were transferred to a solution of bleach containing 5% sodium hypochlorite$^-$ and stirred for 5 min. Seeds were subsequently removed from this solution and washed twice in dH_2O. Seeds were then planted immediately. Storage of seeds is possible for 1-2 days in sealed containers.

2.6.7.2 Sterile Soil Growth

John Innes Soil (John Innes Loam, a monitored soil available from Lincoln University) is pre-packed into split 50 mL Falcon tubes, which were then sealed and wrapped in a rubber coat. Approximately 100 of these tubes were then packed into buckets and sealed, before being gamma irradiated at 25-30000 Gy for 1 h, according to commercial food sterilisation practices at MSD Animal Health (MSD Animal Health New Zealand). This ensured total soil sterility and drastically reduced the handling of soil and subsequent risk of contamination.

Seeds were then sterilised according to the method in **section 2.6.7.1** and then planted in the soil at 2cm depth using sterile tweezers. Dry food storage cereal dispenser boxes of 4.2 L volume (Sistema Plastics) were prepared as growth chambers by removing the top lid and replacing it with 2 layers of Miracloth, packed with cotton wool. This allowed air to enter the chamber whilst filtering out microorganisms. These chambers were then autoclaved before use. Ten mL of sterile H_2O was added to the Falcon tubes, which were then placed into the containers, supported by a sterilised seed tray. The chamber was then sealed and placed in an incubator. Plants were grown for a time period appropriate to each experiment (7 days for microscopy, varying durations for RNA-seq) in a Sanyo MLR-352H humidity controlled incubator using the following conditions for each 24 h period: 10 h maximum light (setting 5), 2 h ‘dusk’ (setting 3), 10 h ‘night’ (setting 0) and 2 h ‘dawn’ (setting 3). Humidity was set at 85% and the temperature was set at 25°C.

2.6.7.3 Spore Isolation and Inoculation of Maize Plants

Spores from *T. virens* Gv 29.8 were obtained from PDA plates after 7 days growth, by suspension in 10 mL dH_2O and filtration through 2 layers of sterile miracloth. Spores were then counted and diluted to the appropriate concentration. Surface sterilised seeds were inoculated with a solution of $1x10^6$ *T. virens* spores in 10 µL of dH_2O. Seeds were air dried in sterile conditions until the water had dried. Seeds were then planted.

2.6.7.4 Hydroponic Growth

Hoagland's basal salt mixture was prepared according **to section 2.3.3**. Media was poured into 50 mL tubes to the 35 mL mark. A plug of sterilised cotton wool was placed into the media so that it was moist but not fully submerged. Seeds were sterilised by the method in **section 2.6.7.1**. As germination was not possible in this media, seeds were germinated for 3 days on sterile germination paper moistened with sterile water before being transferred to the hydroponic solution. Seeds were placed on the centre of the cotton wool, ensuring that they were not fully submerged and then grown for the appropriate duration for each experiment in a Sanyo MLR-352H humidity controlled incubator using the following conditions: 10 h maximum light (setting 5), 2 h 'dusk' (setting 3), 10 h 'night' (setting 0) and 2 h 'dawn' (setting 3). Humidity was set at 85% and temperature was set at 25°C.

2.6.8 Microscopy

Microscopy was carried out using light, fluorescence and confocal microscopes. Both stained and mutants expressing fluorescent mCherry red protein were used to identify *Trichoderma* inside plant tissue. Light microscopes were used at 20x magnification to count spores from protoplast isolations in conjunction with a haemocytometer and for general examination of fungal or bacterial colonies.

2.6.8.1 Staining

Plant roots and fungal material were stained with a combined solution of propidium iodide and wheat-germ agglutinin coupled to Alexa Fluor 488 (WGA - Alexa Fluor 488). Propidium iodide stains the plant cell wall and emits in the red spectrum (excitation 535 nm, emission 617 nm). WGA Alexa Fluor 488 emits in the green spectrum (excitation 495, emission 519 nm). It binds to cell wall chitin of fungi. Prior to staining, roots were preserved and dehydrated in a 3:1 ethanol:acetic acid solution. Clarification of roots was performed by removal from this solution and cooking in 10%

KOH for1 h at 90°C. The sample is then transferred into 1x PBS before application of the staining solution.

Table 9: Propidium Iodide and Alexa Fluor 488 staining solution

Staining Solution	PBS 1x
Propidium Iodide 20 µg/mL	Na_2HPO_4 1.42g/L
WGA Alexa Fluor 488 10 µg/mL	KH_2PO_4 0.245 g/L
0.02% Tween 20	NaCl 8 g/L
in 1 x PBS	KCl 0.2 g/L

2.6.8.2 Fluorescence Microscopy

Fluorescent microscopy was carried out on an Olympus BX51 microscope. Light microscopy was carried out at up to 100x magnification under oil, or 40x without. UV excitation was used to visualise fluorescent samples. Cell^F software was used for digitalisation of images. Green, red and blue filters were available on this microscope. Blue filter (DAPI) excites at 340-380 nm and emits at 435-385 nm. Green filter (GFP) excites at 450-490 nm and emits at 500-550 nm) and red filter (TRITC) excites at 530-560 nm and emits at 572-648 nm.

2.6.8.3 Confocal Microscopy

Confocal microscopy was carried out on a Leica TCS SP5 microscope. Laser excitation was possible between 405-633 nm. Green fluoresce of WGA Alexa Fluor 488 was excited at 488 and detected between 505-560 nm. Red fluorescence was excited at 535 nm and detected at 600-625 nm. Simultaneous detection of these fluorescent emissions was possible and overlay images were created with LAS AF-Lite imaging software. Three dimensional imaging of samples was possible using the Z-stack functionality of this microscope.

3 *Trichoderma virens* is an Endophyte in Cortical Cells of Maize and Inbred Line Affected Root Colonisation

3.1 Abstract

Fungal endophytes must penetrate their host plant and successfully maintain themselves within it. *T. virens* is a plant beneficial fungus that may be endophytic in some plants. This study aimed to confirm the endophytic status of *T. virens* on *Zea mays*. To achieve this, a sterile soil interaction system was created. An assay was then carried out in this system to determine the variability in *T. virens* colonisation ability in different maize lines. The localisation of *T. virens* in its maize host was determined. Microscopic analysis of plants grown in this system was then used to identify structures related to colonisation. After successful colonisation four maize lines had significantly increased colonisation of roots and shoots compared to the remaining 13 lines. *T. virens* was observed forming appressoria and invasive hyphae-like structures during root colonisation, and was seen growing in both intra-cellular and extra-cellular spaces in maize cortical tissue. *T. virens* appeared absent from vascular tissue at 7 days post inoculation (DPI). *T. virens* thus can be endophytic in maize for up to 7 DPI and appears to form infection structures similar to those in plant pathogens.

3.2 Introduction

Trichoderma species are commonly known as plant endophytes, and at this point 12 species have confirmed endophytic capability on various plant hosts (Druzhinina et al., 2011). Despite this, the mechanism behind root colonisation is not well characterised. In *T. harzianum* colonisation of cucumber roots, growth was observed to be predominantly inter-cellular, and was restricted by host cell wall modification (Yedidia, Benhamou, & Chet, 1999). *Trichoderma* spp. usually colonise the outer layers of plant hosts, and are limited to cortical tissue, although one vascular tissue colonising strain has been reported (Harman et al., 2004b; Yedidia et al., 1999). It has also been suggested that *Trichoderma* may form appressoria-like structures, although evidence for these is limited and often during mycoparasitism (Nautiyal & Dion, 2008, p. 244; Omann & Zeilinger, 2010). Structures similar to those used in mycoparasitism (appressoria, haustoria) may play a role in penetration of the plant host (Harman et al., 2004b). Supporting this, *Trichoderma* has been observed using mycoparasitism of mycorrhizal fungi to enter a potato host (De Jaeger, Declerck, & de la Providencia, 2010). Physical penetration may be supplemented or replaced by cell wall

degradation via lytic enzymes. These can either weaken the cell wall, allowing physical entry, or replace physical structures (Murphy, Hodkinson, & Doohan, 2013). It is predicted that *T. virens* will colonise maize root surfaces and cortical tissue and grow in the intercellular space.

The localisation of *T. virens* in its plant hosts is not confirmed. Roots appear to be the primary region *Trichoderma* spp. colonise, although strains colonising leaves and stems have been observed (Harman et al., 2004a; Sundram, 2014). One study of rhizosphere colonisation linked *Trichoderma* growth predominantly to upper root sections and may suggest endophytic colonisation is prevalent in these regions (Cripps-Guazzone, 2014). It is expected that *T. virens* will predominate in root tissue, but may also be found in stems to a lesser extent. Host specificity may play a role in plant-fungal interactions. Specificity of fungal strains to different plant species has been identified in rhizosphere interactions and in several species of endophytes (Cannon & Simmons, 2002; Cripps-Guazzone, 2014; Morán-Diez et al., 2015; Richard S Quilliam, 2012). Recent evidence suggests that host-specificity can be a critical factor – different lines of tomato were shown to affect fungal colonisation of tomato species (Marina Tucci, 2011). The strong host specificity observed in other fungi suggests this may occur in *T. virens* interactions. It is expected that maize line will affect *T. virens* colonisation ability.

To conduct these experiments an interaction system is necessary. The majority of interaction systems use hydroponic growth methods (Djonović, Pozo, Dangott, Howell, & Kenerley, 2006; Kurzbaum, Kirzhner, & Armon, 2014; Morán-Diez et al., 2015). Investigation of root colonisation in soil systems is important in understanding endophytism in conditions more similar to that of the natural environment. The growth media has been confirmed to affect the morphology of colonisation in *Fusarium oxysporum* (Olivain et al., 2006).

This study aims to identify root-penetration structures, to characterise the mode of endophytic growth and to determine the effect of host-line on colonisation. This will be performed in a soil-based sterile interaction system.

3.3 Materials and Methods

3.3.1 Maize Lines – Morphology and Germination

Maize seeds from 15 hybrid lines with different disease resistance traits were generously provided by Pioneer Seed Company and Chieftain Seeds (**Table 2** and **Table 10**). Four pots per maize line

were filled with potting mix (**Section 2.3.3**), and then 10 seeds per line were planted in each pot at 2 cm depth. Pots were watered with 50 mL H_2O. Seeds were grown for seven days and the number of seedlings emerging from the soil was counted.

3.3.1 Development of a Maize-*Trichoderma* Growth System

An interaction system was developed to allow evaluation of plant-fungal interactions. A procedure that retained sterility over the seven day time period proved difficult to establish, and several modifications to the protocol were necessary. The initial protocol was provided by Dr. Natalia Cripps-Guazzone (Cripps-Guazzone, 2014). Growth procedures were tested twice to determine sterility.

The final procedure is presented in **section 2.6.7.2**. The initial procedure and the course of modifications are described below;

3.3.1.1 Original Growth Procedure and Sterility Tests

A large polystyrene block with 50 mL holes in it was sterilised using 70% ethanol and 20 min of UV light treatment (Cripps-Guazzone, 2014). Soil was sterilised using an autoclave and placed in the tray. Maize seeds were surface sterilised in 95% ethanol for 7 min followed by 5 min in 1% bleach. Seeds were planted at a depth of 2 cm and grown for 7 days in an incubator. Root and shoot sections were destructively harvested into 2 cm sections and plated onto PDA. Plates were incubated 7 days at 25°C. If plates were sterile the procedure was accepted.

3.3.1.2 Modifications to Growth Method

Four major modifications to the growth method were made. These were necessitated by the initial contamination detected in the original growth method. The final system was reliably sterile once all of the modifications were made. The initial modification was the replacement of the polystyrene block with a large sealable plastic container to reduce handling difficulty. Secondly, sterile soil was instead prepared by autoclaving three times. Thirdly, seed surface sterilisation was modified to include 5% bleach (Schulz, Wanke, Draeger, & Aust, 1993). Changes were as follows; soil was first pre-packed into 50 mL tubes. Soil was sterilised by gamma ray sterilisation instead of autoclaving. Large containers were replaced by individual sealed containers holding four plants. The modified surface sterilisation procedure (above) was used.

3.3.2 Assay for the Presence and Extent of *T. virens* Colonisation of Maize

An experiment was designed to test whether *T. virens* Gv29.8 was capable of endophytic colonisation of maize, and to simultaneously test the localisation of the fungus in the plant. Fifteen seeds of each maize line described in **Table 2** were surface-sterilised and inoculated with *T. virens* according to the protocols in **section 2.6.7.1**. Seeds were then planted according to the growth protocol in **2.6.7.2**. Maize seeds were planted according to a randomised, double-blind set up which precluded experimental or observer bias. Control plants were, by necessity, planted in their own containers to prevent cross-contamination, but otherwise followed this set up. After 7 days, maize plants were then harvested and surface sterilised in two 2 min washes of 5% bleach. Samples of sterile seeds were plated on PDA to confirm sterility. Sections were then washed again in dH_2O for 2 min. Control plants were sectioned and washed first. Water and bleach were changed every 5 min. Plants were then cut into sections according to **Figure 2** . Sections were then plated onto TSM (**section 2.3.2**) and grown for 7 days at 25°C. If any controls were contaminated, the experiment was discarded. This allowed determination of both the presence of *T. virens* and its most common point of detection in this interaction system. Statistical significance of the data was measured using a generalised linear model (GLM) statistic

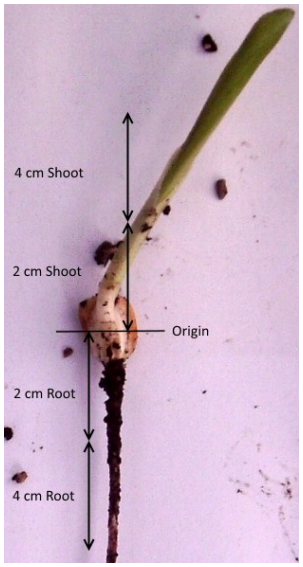


Figure 2: Diagram of root and shoot sections used for *T. virens* endophytism study.Maize roots were cut into 2 cm sections with the seed as the point of origin for measurement.

Table 10: Disease resistance traits for maize lines used in this study.

Disease or Maize Line	33M54	34F95	34H31	34K77	35A30	36B08	36M28	37Y12	38V12	39G12	39T45	P0537	F1	G30	Popcorn
Northern Leaf Blight	5	6	6	5	6	7	5	6	6	4	6	6	N/A	N/A	N/A
Common Rust	6	6	4	6	6	5	7	5	6	6	6	6	N/A	N/A	N/A
Eyespot	N/A	N/A	N/A	6	6	6	N/A	6	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Head Smut	N/A	7	5	7	N/A	8	7	6	N/A	8	N/A	N/A	N/A	N/A	N/A
Fusarium Ear Rot	N/A	6	5	6	N/A	5	5	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Diplodia Ear Rot	N/A	4	5	3	N/A	4	N/A	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Giberella Ear Rot	N/A	5	4	5	N/A	5	5	5	5	5	N/A	N/A	N/A	N/A	N/A
Anthracnose Stalk Rot	N/A	5	5	4	N/A	5	5	4	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Maize Hybrid Line traits for disease resistance are shown here (Data obtained from Pioneer Seeds). Resistance scores are arbitrary rating (out of 10, 10 is most resistant) assigned to a line based on their resistance to a pathogen (column 1). Higher scores indicate superior resistance to a pathogen. N/A means that data was unavailable for these lines.

.

3.3.3 Microscopic Analysis of *T. virens* in Maize host

3.3.3.1 Fluorescence Microscopy

Maize inbred line 34H31 seeds were inoculated with *Trichoderma* and grown according to the protocol described in **section 2.6.7.2**. At 7 DPI the entire plant was removed from each tube, washed and then placed in a 50 mL tube. A 2 cm root section was cut from each plant from the point closest to the seed. Root sections were dehydrated, clarified, stained and examined under the fluorescent microscope by the methods described in **section 2.6.8**.

3.3.3.2 Microscopy Using Fluorescently Labelled Mutants

Trichoderma virens mutants expressing the mCherry (Shaner et al., 2004) fluorescent protein (Shaner et al., 2004) were obtained from Artemio Mendoza-Mendoza. Colonies of the mutants were grown on PDA for 7 days and the spores harvested by filtration through 2 layers of sterile Miracloth. One million spores were inoculated per seed, which were then planted according to the hydroponic growth protocol (**Section 2.6.7.4**).

Seedlings from maize inbred line 34H31 plants were harvested destructively and gently sectioned into 2 cm pieces, which were then placed on microscope slides and examined under the fluorescence microscope as described in **section 2.6.8**.

3.3.3.3 Confocal Microscopy

Maize plants (line 34H31) were grown and inoculated with *T. virens* Gv29.8 according to both the standard growth protocol and the hydroponic growth protocol. Root sections were cut from plants at 7 DPI and prepared as described in **section 2.6.8**.

3.3.3.4 Transmission Electron Microscopy

Samples for transmission electron microscopy were grown according to the standard growth method (**section 2.6.7.2**) and then placed in a 25% glutaraldehyde, 2% formaldehyde solution in 0.1 M phosphate buffer at pH 7.4 for 2.5 hrs. Samples were then transferred to a 0.1 M phosphate buffer solution until treated. Treatment consisted of 4 h in a 1% OsO_4 solution in 0.1 M phosphate buffer, followed by overnight storage in the same buffer. A 1% tannic acid solution was applied for 2 h followed by 1.5 h in 0.1 M phosphate. Finally, the sample was placed in a 1% sodium sulphate solution for 15 min before three five min washes in H_2O. Transverse sections were created by Dr.

Duane Harland (AgResearch) using a glass knife. Immediately before examination a series of 1 h acetone treatments (70-100%) were performed. This was followed by application of 50% procine 50% acetone solution for 4 h. Sections were then observed under a transmission electron microscope.

3.4 Results

3.4.1 Germination Test

Maize lines in the growth trials had a germination rate above 90% with the exception of Chieftain F1, popcorn and G30 **(Data not shown)**. Root numbers varied between 1 and 5. Chieftain F1 and popcorn had the lowest number of germinating plants.

3.4.2 Development of the Maize-*Trichoderma* Interaction Systems

Various maize interaction systems described in **section 3.3.1** were tested to determine sterility, by plating control seeds on PDA. Control sterility improved as modifications to improve sterility were implemented in the method. The final method maintained sterility and was therefore used for further experiments (**Table 11**).

Table 11: Sterility of maize control plants in various-*Trichoderma*-Maize Interaction systems

Control Plants	Original Method	Modification 1	Modifications 2 and 3	Final Method
Contaminated	26	20	14	0
Total	30	30	30	30

3.4.3 *T. virens* Colonisation of Maize Lines

Trichoderma virens ability to colonise the roots and shoots of 15 different maize lines was examined by plating assay based on TSM (Elad, Chet, & Henis, 1981). Roots were colonised more extensively and reliably than shoots during the 7 days of the experiment (**Figure 3**). Root colonisation progressed at least 4 cm in all lines except F1 and 39G12. Shoot colonisation only progressed past 2 cm in half of the maize lines. The primary localisation of the fungus after 7 days was in the 2 cm section of root directly below the seed, closely followed by the 2 cm shoot section above the seed.

Table 12: Statistical analysis of maize root and shoot colonisation

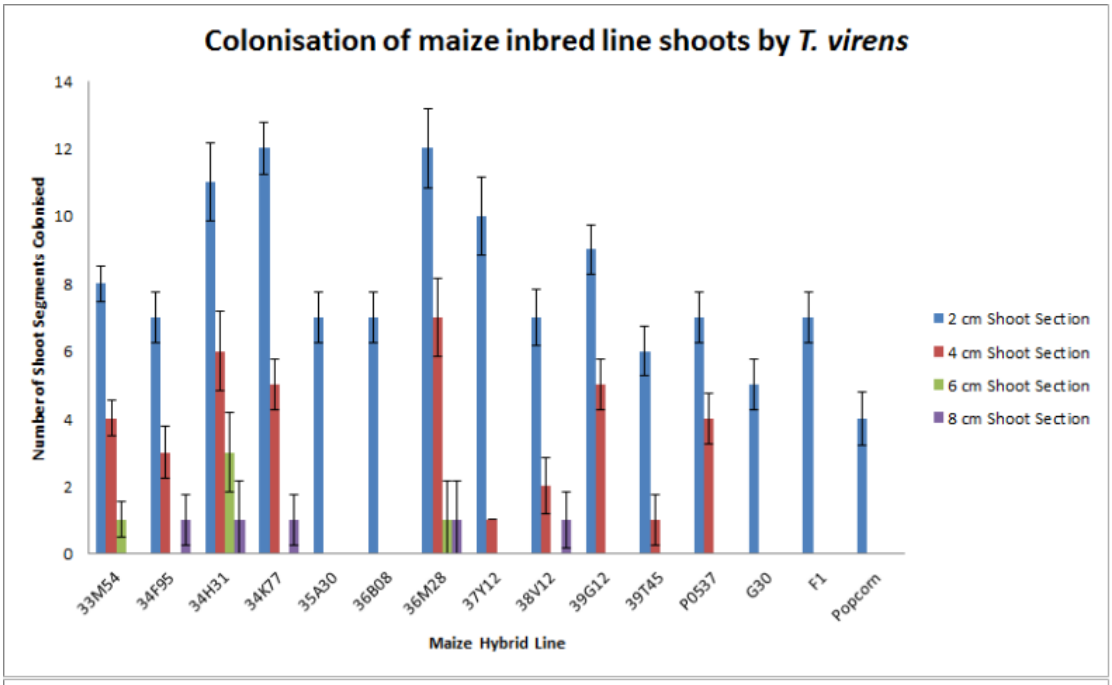
Line	Estimate	Std. Error	z value	Pr (>|z|)
Line 33M54	-0.4055	0.527	-0.769	0.4417
Line34F95	1.0986	0.7601	1.445	0.14836
Line34H31	3.0445	1.1615	2.621	0.00877 **
Line34K77	1.0986	0.7601	1.445	0.14836
Line35A30	0.2719	0.7387	0.368	0.71277
Line36B08	-0.2877	0.7601	-0.378	0.70508
Line36M28	3.0445	1.1616	2.621	0.00877 **
Line37Y12	3.0445	1.1616	2.621	0.00877 **
Line38V12	1.7918	0.8333	2.15	0.03155 *
Line39G12	0.539	0.7387	0.73	0.46558
Line39T45	0.8109	0.7453	1.088	0.2766
LineF1	-0.2877	0.7601	-0.378	0.70508
LineG30	-0.2877	0.7601	-0.378	0.70508
LineP0537	0.8109	0.7453	1.088	0.2766
LinePopcorn	-0.6061	0.7866	-0.771	0.44093

Standard deviation for this data was 2.85×10^{-6}

Variation between the ability of *T. virens* to colonise each line was quantified using a generalised linear model (GLM). Root and shoot colonisation were significantly affected by maize line (**Table 12**)**, p =** 0.01 (3 lines) and **p**= 0.05 (38V12)) at 7 DPI. Four of the 15 maize lines were significantly more susceptible to *T. virens* colonisation. These were 34H31, 36M28, 37Y12 and 38V12, in all of which both root and shoot colonisation were significantly higher than in other lines. There was no detectable significant difference between root and shoot colonisation at the 7 DPI time point.

3.4.1 Microscopic Analysis of *T. virens* Root Colonisation

Trichoderma virens was observed growing extensively on root surfaces and outer cell layers (**Figure 4**). Hyphal growth originated from spores adhering to the root surface, or from longer hyphae of spores proximal to the inoculation site. Hyphae also appeared to commonly grow in a manner that followed plant cell wall spaces.



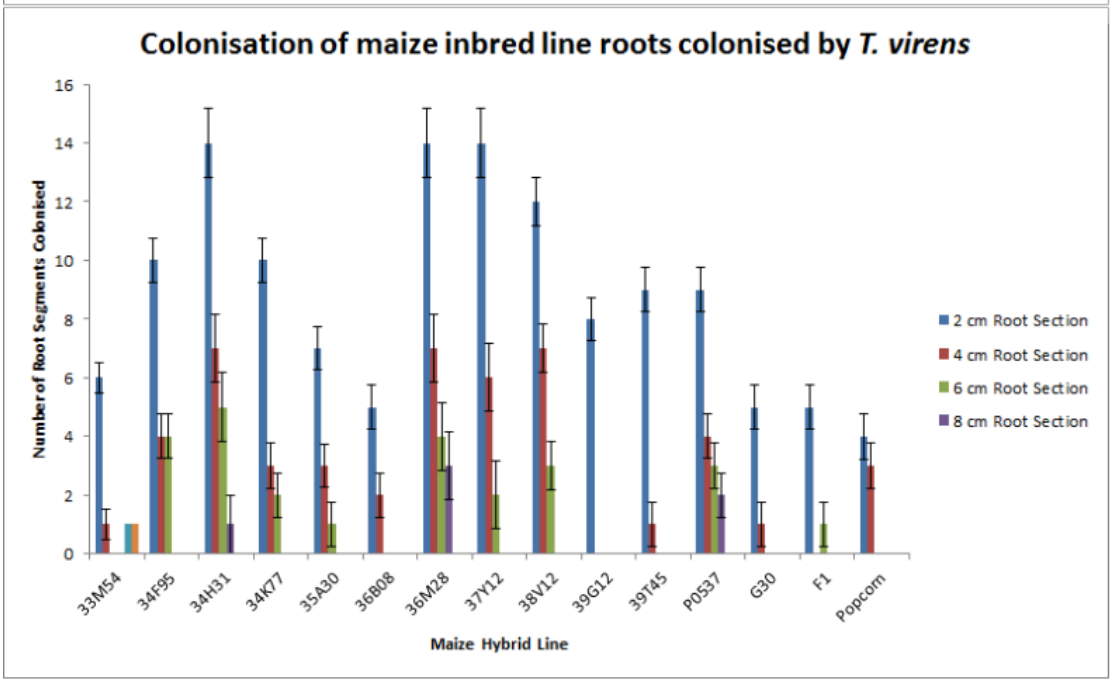


Figure 3: The incidence and extent of maize inbred line 34H31 root and shoot colonisation by *T. virens* Gv 29.8.The number of individual maize plants of each hybrid line that were colonised by *Trichoderma virens* after 7 days. Significance of data was determined using GLMs.

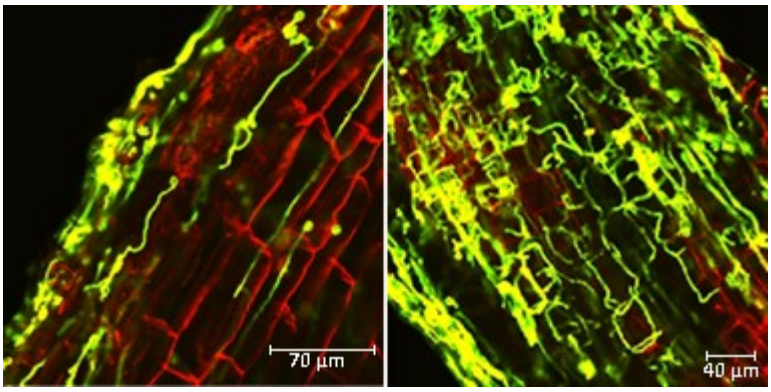


Figure 4: Surface colonisation of maize roots by *T. virens*.*T. virens* was stained in green with WGA Alexa-Fluor 448. Maize cells are stained with propidium iodide. This image was taken on a Leica laser confocal microscope.

Cellular penetration by appressorium-like structures was observed, albeit not as commonly as predicted (**Figure 5A**). A haustorium-like structure (**Figure 5B**) was captured inside the same maize cell, with hyphae linking it to the penetration peg. This indicates intra-cellular growth is possible by *T. virens* in a maize host.

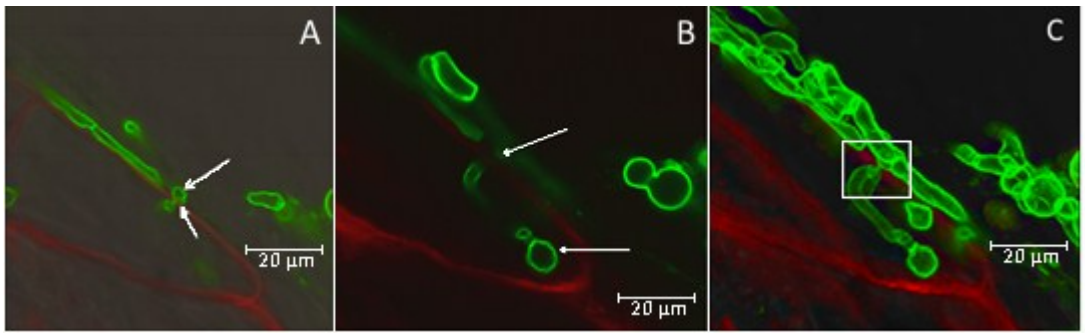


Figure 5: Formation of appressorium and haustorium-like structures on maize tissue by *T. virens*.Images were captured at 100x magnification using the Leica LCS5 confocal microscope. Fungal material was stained as with WGA-Alexa Fluor 488 and plant material was stained by propidium iodide (see **section 2.6.8.1**). Image **A** shows the appressorium-like structure and the peg crossing the plant cell wall surface. Image **B** shows a haustorium-like structure and its location relative to the penetration site. Image **C** is maximum projection of this site created across several focal depths. Plants were grown in hydroponic systems.

A second mechanism of colonisation was identified, with hyphae that had forced themselves between maize cells being observed (**Figure 6**). Hyphae growing in areas where maize cell walls were pushed out of tight contact were seen in outer cell layers and, more rarely, seen deeper in cortical tissue. No fungus was observed in vascular tissue at any stage. Control plants did not show similar separation in cell walls (**Data not shown**).

Transmission electron microscopy (TEM) identified small (1-2 µm) melanised structures, which resemble structures observed in *Aureobasidium pullulans* (Atsatt & Whiteside, 2014). These

structures have been observed inside plant cells and organelles, such as chloroplasts. *T. virens* inoculated maize plants contained these structures most often in association with the cell wall, but also within host cells (**Figure 7**). These melanised cells do not stain with chitin binding fluorescent stains in other fungi, however once outside plant cells are capable of reverting to stainable structures (Atsatt & Whiteside, 2014).

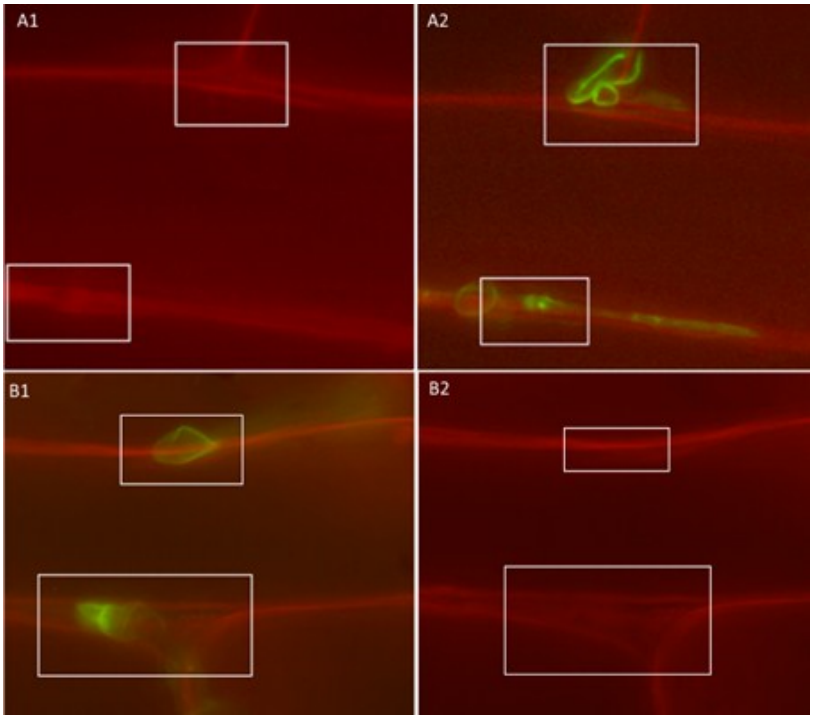


Figure 6: Extra-cellular endophytic growth of *T. virens* forces maize cells apart. *Trichoderma virens (*green) is shown growing between cells in the intra-cellular region of soil-grown maize. The boxes highlight identical areas in each image pair. **A1** and **A2** show hyphal growth in the areas being forced apart in the maize cell. **B1** and **B2** show a similar pattern of growth from a different maize root. *T. virens* is co-localised with regions where the cells are forced apart. Fungal material was stained as with WGA-Alexa Fluor 488 and plant material was stained by propidium iodide (**see section 2.6.8.1**).

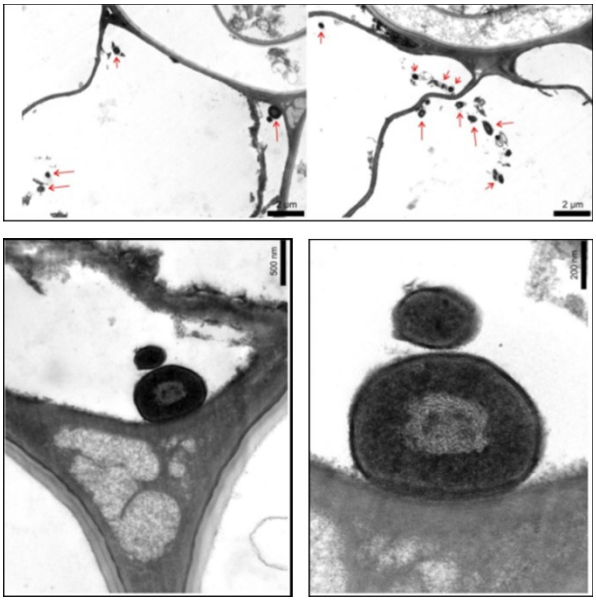


Figure 7: Transmission electron micrograph of *T. virens* inoculated maize plants showing small melanised structures. These structures (indicated by red arrows) bear resemblance to miniature hyphae that have been observed in other endophytes. However these did not stain with Alexa Fluor 488, indicating that either they were too deep for the dye to penetrate, or that the cell wall structure of the micro-hyphae is different to that of regular hyphae.

3.5 Discussion

The establishment of a reliable interaction system was crucial to the success of the experiments in this chapter and the ability to isolate the maize-*Trichoderma* interaction was essential for monitoring endophytic behaviour in the fungus. Soil is rarely used in molecular systems; however it has been reported that phenotypes observed on hydroponic systems differ from those in soil (Olivain et al., 2006). Maintaining sterility in soil systems proved difficult and this was overcome predominantly by gamma-ray sterilisation, suggesting that the soil itself was the major issue. Phenotypic variation was observed in *Trichoderma* between soil and hydroponic systems (**data not shown**), probably due to the increased osmotic stress and different nutrient profiles of soil and Hoagland's media. The hydroponic system's advantage was the cleaner root sections and reduced damage to root systems. This system was also applicable to other experiments such as RNA-sequencing.

The maize-colonisation assay indirectly confirmed endophytism of *T. virens* in *Z. mays*. Every maize line in the study was colonised by *T. virens* and 4 maize lines with significantly more colonisation than the other lines were identified. This result was expected, as the disease resistance attributes of each line varied (**Table 10**) although no clear correlation between these resistance traits and the

maize line susceptibility to *Trichoderma* was detected. Plant morphology may have had some effect on colonisation, as lines with single tap roots appeared to show reduced colonisation (Popcorn, G30, F1). Similar studies in tomato also support this evidence of host line-specificity (Marina Tucci, 2011). Maize line specificity suggests that differences in host genomes may have wider effects on root colonisation. This experiment has interesting implications for agricultural use of biocontrol agents, in that it may be necessary to match plant hybrid lines to specific biocontrol strains. Repetition of this experiment with a greater plant population is recommended to expand upon this result and enhance its statistical resolving power. This result is consistent with the concept of avirulence genes – R gene relationships in fungal pathogens, where single genes commonly cause avirulent phenotypes on a particular host (Cai, Xu, & Zheng, 2002; Cannon & Simmons, 2002; Laugé & De Wit, 1998). The colonisation of all lines suggests that either the immune response of each is not entirely successful at preventing *T. virens* colonisation, or that insufficient time was available for the fungus to be completely cleared from the plant. Persistence of *Trichoderma* species in plant hosts for up to 5 weeks has been reported, so clearance may have been outside the time-scale of this investigation (Sobowale et al., 2007).

Morphological structures were identified with strong resemblance to appressoria and invasive hyphae (**Figure 8**). These are commonly associated with biotrophic feeding and effector secretion in pathogenic fungi (Giraldo & Valent, 2013; Staples, 2001; Szabo & Bushnell, 2001). The association of the appressorium with an invasive-hyphae like structure observed in this study resembles the models proposed in the plant pathogens *Magnaporthe oryzae* and *Colletotrichum higginsianum* (Djamei & Kahmann, 2012; O'Connell et al., 2012). However, such structures were not observed as commonly as would be expected if they were a primary mechanism of root penetration. Despite this they may form an important interface for effector delivery, although further evidence would be required to establish evidence of this biochemistry. The detection of putative micro-hypae-like structures by electron microscopy may provide some explanation for the low number appressoria visualised, as they may be capable of growing between cells.

The limited growth of *T. virens*, in only cortical tissue is intriguing. Bacterial endophytes are capable of colonising vascular tissue, and use it to facilitate systemic colonisation (Germaine et al., 2004; Shishido, Breuil, & Chanway, 1999). Pathogenic organisms associated with vascular colonisation tend to be associated with severe symptoms, although such vascular growth and the associated symptoms may only be triggered at certain times in the plant or fungal life cycle (Yadeta & J.

Thomma, 2013). The ability of maize to exclude *T. virens* from vascular tissue may be important in maintaining a mutualistic relationship, or limiting the extent of colonisation.

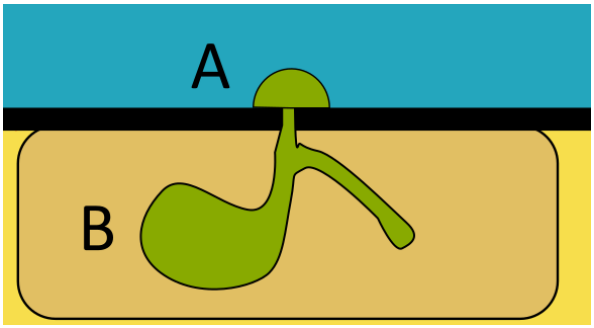


Figure 8: A diagram of the model appressoria and invasive structure in fungi The diagram shows penetration of a host cell using appressoria (**A**). This forces the penetration peg into the cell, where invasive hypae or haustoria form (**B**) and can engage in nutrient acquisition and effector delivery.

Proliferation of *T. virens* in the plant most likely occurs by surface or outer-cell layer colonisation. *T. virens* growth was extensive on the outer root surface, and may be the primary mechanism of spread along the root length. It may also explain the frequency of colonisation in the outer two layers of the plant cortex. *T.* virens growth in this cortical region was common in inter-cellular spaces. This behaviour has been observed in other *Trichoderma* species (Yedidia et al., 1999). Expression of swollenin, which is responsible for cell wall loosening and enhance root colonisation has been shown in *Trichoderma reesei* and may explain this observation (Brotman et al., 2008; Saloheimo et al., 2002). Electron microscopy identified small hypha-like structures, which may constitute a novel method of proliferation or nutrient acquisition. However, *T. virens* predominated on plant surfaces, supporting the suggestion of 'opportunistic endophytism' (Harman et al., 2004b).

3.6 Conclusion

The plating assay and microscopic observations both confirm that *T. virens* can be endophytic in various maize lines. Direct microscopic evidence in maize line 34H31 showed both intra-cellular and inter-cellular growth is possible. Fungal growth appears to spread initially across the root surface, with limited endophytic growth occurring in the cortical layers. Although appressoria and invasive hyphae-like structures were visualised, these were relatively uncommon, with inter-cellular growth being the primary mechanism of root colonisation. This aligns with previous observations of *Trichoderma* spp., although intra-cellular growth has not previously been observed (Harman et al., 2004b; Yedidia et al., 1999). Appressoria formation did not appear to be common enough to explain the extent of inter-cellular growth by *T. virens*. This suggests an alternate method of root entry such as enzyme based cell wall degradation.

4 Bioinformatic Analysis of Three *Trichoderma* Genomes Identified Putative Effectors and Functional Groups Relevant to Root Colonisation

4.1 Abstract

The mechanisms by which effector proteins modulate the innate plant immune response are quite diverse and include the inhibition of chitinases, proteases, reactive oxygen species (ROS)-scavenging, as well as direct modulation of host transcriptional machinery. Few known *Trichoderma* effectors exist however, chitin scavenging proteins such as LysM have been shown to suppress plant responses to chitin. Other small secreted proteins, such as Sm1, are known to affect plant immune responses. This chapter describes and compares potential effector proteins in three different *Trichoderma* species. Several criteria were used to identify effectors in *T. atroviride* (IMI206040), *T. reesei* (QM6a) and *T. virens* (Gv29.8), including the existence of classical signal peptides, an amino acid length under 300 amino acids ('small'), and relatively high cysteine content (5% or more). Protcomp analysis was used to determine potential effectors via cellular localisation signals. Tandem repeat analysis was performed, as proteins with numerous tandem repeats have been determined to have effector activity. Examples of this include bacterial transcription activator like effectors (TAL) and fungal LysM proteins. A total of 2,676 putative secreted proteins were identified in the three genomes analysed here, 677 of which do not have any evident motifs. Several groups of putative effector proteins, including ubiquitin ligases, protease inhibitors, and transcription factors were detected. The fungus with the highest diversity of effectors was *T. atroviride,* closely followed by *T. virens*. *Trichoderma reesei* had fewer putative effectors, possibly reflecting its more specific saprobic lifestyle.

4.2 Introduction

Most plants are colonised by fungi that cause no disease symptoms. Endophytes can improve plant growth, disease resistance, and abiotic stress tolerance, while the fungi themselves obtain nutrients (Rodriguez, White, Arnold, & Redman, 2009). Several strains of the soil-borne fungus *Trichoderma* establish an endophytic relationship with roots of economically important crops such as maize and tomato, improving their fitness (Alfano et al., 2007; B. A. Bailey et al., 2006; Chacón et al., 2007; Harman et al., 2004b; Shoresh & Harman, 2008; Shoresh et al., 2010; Yedidia et al., 1999). Despite

directly benefiting from this symbiosis, plants still react to colonisation from endophytes by activating their innate immune system (Rodriguez et al., 2009; Shoresh et al., 2010; Yedidia et al., 1999), which has evolved to recognise common features of microorganisms (Jones & Dangl, 2006). Plants translate this recognition into a defence response that is specifically directed against the invader encountered (Bittel & Robatzek, 2007; Jones & Dangl, 2006; Schäfer et al., 2009). Endophytes must overcome the plant's immune responses in order to successfully penetrate a cell. This is achieved by reprogramming the invaded cell to accommodate infection structures of the fungus, and to maintain host cell integrity for long-term symbiotic interactions (B. A. Bailey et al., 2006; Chacón et al., 2007; Djonovic et al., 2007; Shoresh et al., 2010; Van Wees et al., 2008; Yedidia et al., 1999).

Plant pathogenic bacteria, fungi, oomycetes, and nematodes must overcome the same obstacles as endophytes and therefore endophyte effectors may follow similar mechanisms. This is supported by the presence of effector proteins, similar to those in pathogens (Schäfer et al., 2009; Van Wees et al., 2008), in the mutualistic root symbionts *Piriformospora indica* (Zuccaro et al., 2011) and *Laccaria bicolor* (F. Martin et al., 2008). Pathogens manipulate and evade plant immune responses by delivering an arsenal of these effector molecules into their hosts (Hogenhout, Van der Hoorn, Terauchi, & Kamoun, 2009; Kamoun, 2007). Plants have evolved receptors (R proteins) to sense the activity of some of these pathogen effectors and trigger strong defences (Jones & Dangl, 2006; Yedidia et al., 1999). The ensuing battle between pathogens and plants is a series of measures and counter-measures, which involve activation and repression of many genes in both partners (Birch et al., 2009; Li et al., 2010). The recently released genomes of the mycoparasitic and endophytic fungi, *T. atroviride* and *T. virens* (Kubicek et al., 2011), and the saprobic fungus *T. reesei* (Martinez et al., 2008), will shed some light on the molecular mechanisms occurring during plant-*Trichoderma* interaction. In this section, the presence of molecules with effector activity in *Trichoderma* spp. were identified by a bioinformatics approach.

Effectors are secreted molecules that alter plant processes and facilitate colonisation, either in plant or animal pathogens (Hogenhout et al., 2009; Kamoun, 2007). The literature suggests that effectors may be small (300 amino acids or less), cysteine-rich proteins that may contain a pathogenesis motif, a nuclear localization signal, or any domain that is atypical of secreted proteins of known function (e.g. transcription factors, ubiquitin ligases), and may also contain internal repeats (Saunders et al., 2012).

Effectors may be localised to different target areas in the host plant (**Figure 9**). Apoplastic effectors are often small, highly cysteine-rich proteins that contain intra-molecular disulfide bridges, most likely to maintain stability in harsh environments such as the plant apoplast. Apoplastic effectors are variable, and some have been identified as serine and cysteine protease inhibitors that target host proteases (Doehlemann et al., 2009; Shabab et al., 2008) or minimise the levels of ROS (Dong et al., 2011; Stergiopoulos et al., 2010), and protect fungal cell walls against hydrolysis by plant chitinases (Stergiopoulos et al., 2010) and phytotoxic proteins. Several apoplastic proteins may be involved in masking fungal detection by the plant by operating as protectors of fungal proteins(de Jonge et al., 2010; de Jonge & Thomma, 2009). LysM, for example, is thought to operate by scavenging chitin molecules, thus the ability of the plant to detect and respond to the fungus (de Jonge & Thomma, 2009).

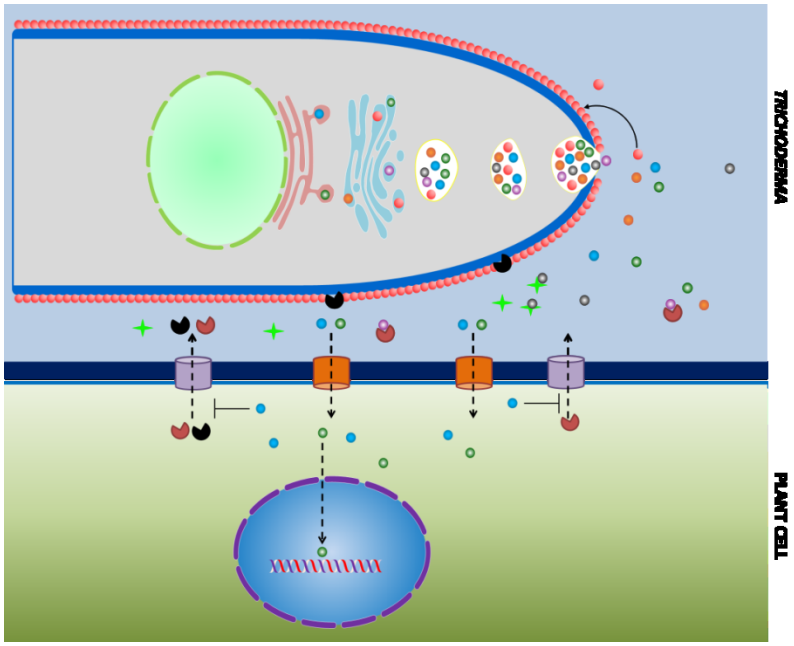


Figure 9: Secretion of effector-like proteins during the plant-*Trichoderma* interaction. The effector-like proteins are secreted via the type II secretion system. Effectors are released from fungal vesicles into the cytoplasm, where they may directly interact with host defence proteins. Some effectors may interact with plant cell surface proteins, or be directly translocated into the host plant, where they can regulate host processes and transcriptional machinery. Image kindly provided by Artemio Mendoza.

Cytoplasmic effectors may have a different mechanism to apoplastic effectors. Gram negative bacteria release effector proteins directly to the plant cytoplasm using a molecular syringe called the type III secretion system (T3SS) (Koeck, Hardham, & Dodds, 2011). The type III secretion signal located in the N-terminus seems to be universal and conserved among animal and plant pathogens as well as plant symbionts (Hajri et al., 2009). Nematodes use their stylet to puncture the plant,

delivering effector proteins into the apoplast or the cytoplasm of plant cells (Rosso, Vieira, de Almeida-Engler, & Castagnone-Sereno, 2011). By contrast, very little is known about how filamentous pathogen effectors are released into the plant host cell. Fungal effectors may also be secreted in the hyphal tip using the conventional secretion signal type II (Kamoun, 2006; Oliva et al., 2010). The majority of filamentous pathogen effectors identified to date carry typical signal peptides (Choi et al., 2010; Kamoun, 2006, 2007). Effectors with potential cytoplasmic localisation domains have been reported in rust fungi (Saunders et al., 2012). However, the domains responsible for such translocation have not been identified in other fungal groups. By contrast, effectors from oomycetes that act inside the host cell often contain a conserved host-translocation motif, which is essential for their translocation to the cell. This motif, composed of the sequence RxLR-EER, appears to be been extended and conserved in oomycetes, including *Phytophthora* spp. (Birch et al., 2009). Other motifs, such as the WxxxE, KRKR and Lx2Rx4L are involved in bacterial effectors, but may be limited to the type three secretion system (Dean, 2011; Wallenfang, 2009).

The molecular mechanisms that govern the immune response to *Trichoderma* are still largely unknown. ISR and plant colonisation are, however, known to be independent mechanisms, since *Trichoderma* spp. mutants that are unable to induce ISR are still able to colonise roots at similar rates to wild type *Trichoderma* spp. (Viterbo, Harel, Horwitz, Chet, & Mukherjee, 2005). ISR is now known to be dependent on the plant hormones jasmonic acid (JA) and ethylene (ETH) (Bittel & Robatzek, 2007; Schäfer et al., 2009; Van Wees et al., 2008). Enhanced protection against reactive oxygen species (ROS), and repression of the ethylene synthesis pathway, is proposed to enable root colonisation by *Trichoderma* as has been shown in other endophytes (Shoresh et al., 2010). Several biochemically diverse MAMPs have been identified in *Trichoderma* (Djonovic et al., 2007; Djonović, Pozo, Dangott, et al., 2006; Shoresh et al., 2010), including the hydrophobin-like protein Sm1 (Djonovic et al., 2007) and the ethylene-inducing xylanase (EIX) (Ron & Avni, 2004). The hydrophobin is induced during plant interaction with the fungus, and promotes the expression of pathogenesis-related genes and hypersensitive reactions (Djonovic et al., 2007). EIX has a dual role during plant colonisation, involving both lytic enzyme activity and the induction of systemic resistance in specific cultivars of tobacco and tomato (Bar, Sharfman, Ron, & Avni, 2010, p. 1; Hanania, Furman-Matarasso, Ron, & Avni, 1999; Ron & Avni, 2004; Rotblat, Enshell-Seijffers, Gershoni, Schuster, & Avni, 2002). The role of effector proteins or other biochemically active small proteins in the interaction may therefore be significant, and detection of these is the focus of this chapter.

4.3 Methods

Full proteome datasets for *T. virens* (Gv 29.8)*, T. reesei* (QM6a) and *T. atroviride* (IMI206040) were obtained from the JGI website and the secretome of these species was identified (**Figure 10**) [http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html, http://genome.jgi-psf.org/Trire2/Trire2.home.html and http://genome.jgi.doe.gov/Triat2/Triat2.home.html, respectively]. Datasets were divided to conform to the maximum size limit allowed by the CBS SignalP 4.0 server [http://www.cbs.dtu.dk/services/SignalP/] (Petersen, Brunak, von Heijne, & Nielsen, 2011). Proteins containing a SignalP sequence that also contained no trans-membrane domains were screened using R scripts (**Supplementary Material**) and inserted into Fasta files. TMHMM and Protcomp [http://linux1.softberry.com/berry.phtml?topic=protcompan&group=programs&subgroup=proloc] were used to confirm the absence of trans-membrane domains, GPI-anchors and transit peptides of mitochondria and chloroplasts. Alignments and manual searches, using MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) and R scripts, were used to manually analyse datasets for size distribution and known effector motifs (e.g. RxLR). The dataset was then split into approximately 60000 character datasets to conform to the limits of MEME analysis (T. L. Bailey et al., 2009). MEME was used to detect novel motifs with significance over e-20 *de novo*. The following search parameters were used: 4-10 amino acid motif length, zero-one motifs per sequence in order to maintain consistency with searches performed in the literature (Saunders et al., 2012). Sequences containing motifs were then analysed using Basic Local Alignment Search Tool (BLAST) (Altschul, Gish, Miller, Myers, & Lipman, 1990), implemented in Blast2Go and ELM (Van Roey et al., 2014) to detect homology to known proteins. Sequences with homology to known proteins with enzymatic activity were removed from the database. Jalview 2.7 and Excel [http://www.jalview.org/] were used to create graphs of the data. Cysteine-rich proteins were identified by sorting sequences under 300 amino acids in length by size, and then calculating the percentage of cysteine residues using R scripts. Cysteine content over 5% was considered to be 'cysteine rich' Graphs were constructed in Excel. Tandem repeats were identified using X-stream.

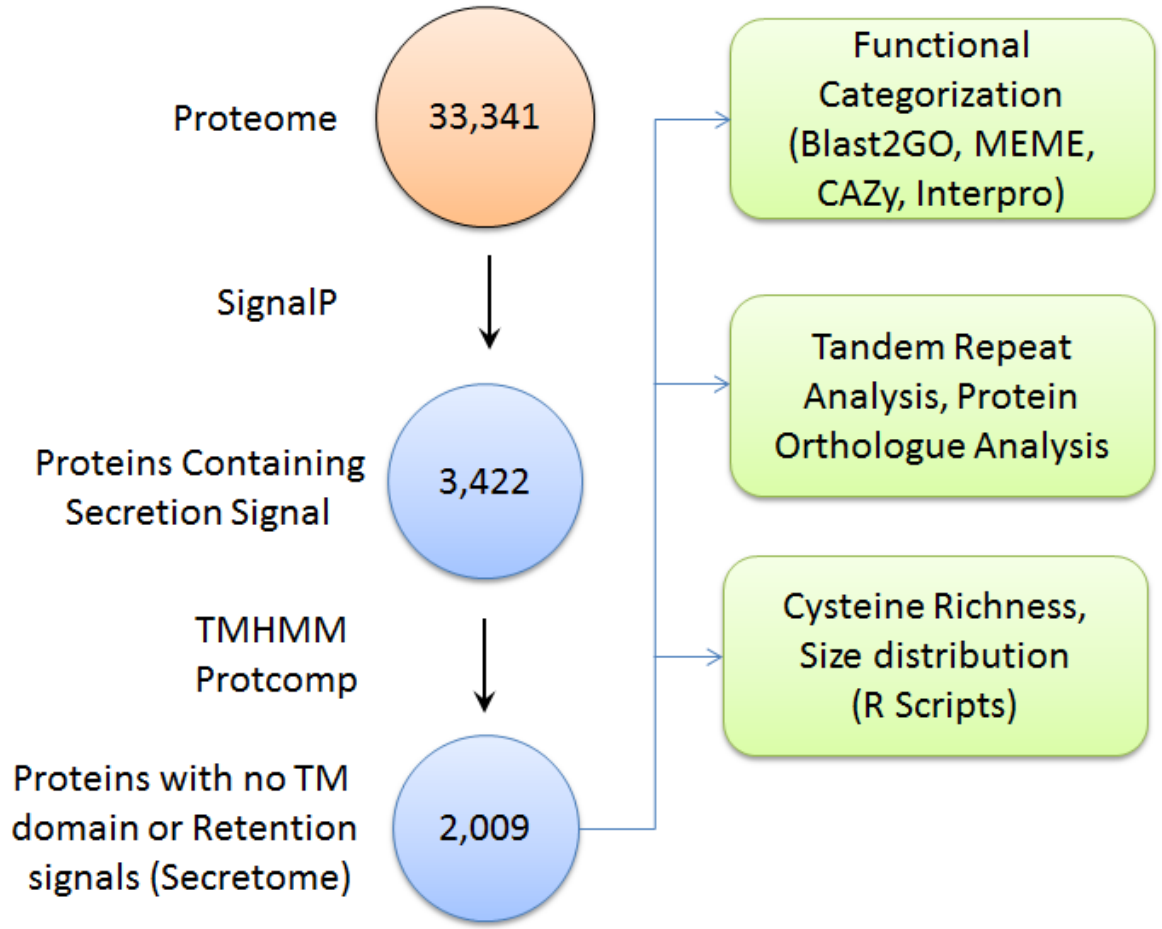


Figure 10: Methodology for secretome Identification by bioinformatics A diagrammatic outline of the process used to detect and analyse putative small secreted proteins.

4.4 Results and discussion.

4.4.1 The Secretome of *Trichoderma* spp.

To identify potential effector molecules secreted by *Trichoderma* spp., the secretome of two endophytic *Trichoderma* species, *T. atroviride* and *T. virens*, and the saprobic fungus *T. reesei* was investigated. Of the total proteome of these organisms, nearly 9.0 % of proteins contained a potential secretion signal (3,422 of 33,435 proteins in the three species) (**Table 13**). Of these, 58.7% were proteins that did not contain a trans-membrane domain and were potential secreted proteins. Cell membrane anchored effectors or effectors with non-standard secretion signals may also exist, and would not have been detected by this method. Indirect evidence of this exists in a number of homologous, potentially secreted, proteins that had no detectable secretion signal. For example,

TV_213200 contains a thaumatin domain (IPR001938) and is highly similar to the putatively secreted thaumatin-like TV_70385, yet it contained no known secretion signal.

Table 13: Number of potential effectors compared to the proteome sizes of the three strains of *Trichoderma*

Dataset	*T. atroviride*	*T. virens*	*T. reesei*
Total Proteome	11865	12427	9143
Signal P4	1195	1267	960
Secretome	729	726	554
Tandem Repeats*	102	108	100

The total proteome size of the three *Trichoderma* strains is shown. *Trichoderma virens* has the largest proteome size. *T. atroviride* and *T. virens* have approximately 25% more effectors than *T. reesei,* which probably correlates with the saprobic lifestyle of *T. reesei*. * - Large tandem repeat proteins may act as effectors by delivering multiple copies of smaller repeat proteins.

The distribution of secreted proteins between species appeared to be relatively similar, although *T. reesei* had a slightly smaller secretome. Analysis of the size distribution of these proteins, found that the majority of secreted proteins fall between 100-500 amino acids in length (**Figure 11)**. Slightly over half of all secreted proteins were above the 300 amino acid limit assigned for small secreted proteins. The largest putatively secreted protein was 3241 amino acids in length. This may suggest that several proteins above the usual size considered for effectors may be relevant to plant-fungus communication.

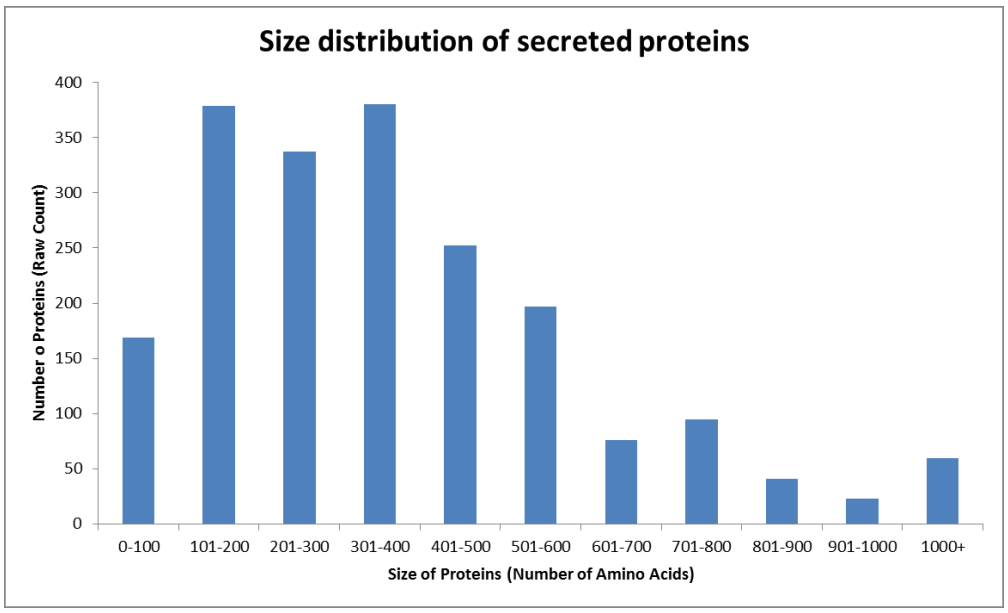


Figure 11: Length distribution of secreted proteins. The majority of secreted proteins fall between 100 and 600 amino acids in length. This is slightly larger than the length predicted for effectors in the literature (fewer than 300 amino acids).

4.4.2 Small Cysteine-rich Secreted Proteins

The three *Trichoderma* species contained 827 proteins under 300 amino acids in length identified by SignalP. Protcomp analysis identified 655 small secreted proteins. Of the 827 proteins, 430 had no matches to proteins of known function (**Supplementary Table 2**). Only 129 proteins were specific to only *Trichoderma* species, and eight were unique to a single species. Of these, 173 contained over 5% cysteine (169 in Protcomp dataset, **Figure 12**). Protcomp varies from SignalP in that it also detects cellular localization, and thus allows removal of proteins unlikely to be effectors, such as those in the mitochondria.

BLAST analysis of the small secreted proteins showed that a large number of these proteins were specific to *Trichoderma* species. *Trichoderma atroviride* in particular had a considerable number of proteins with low homology to known proteins. Around 25 of the 75 small cysteine rich secreted proteins analysed had only one BLAST match. Twenty-four of these were from *T. atroviride*, indicating that it potentially has a greater range of novel cysteine rich effectors than the other two species.

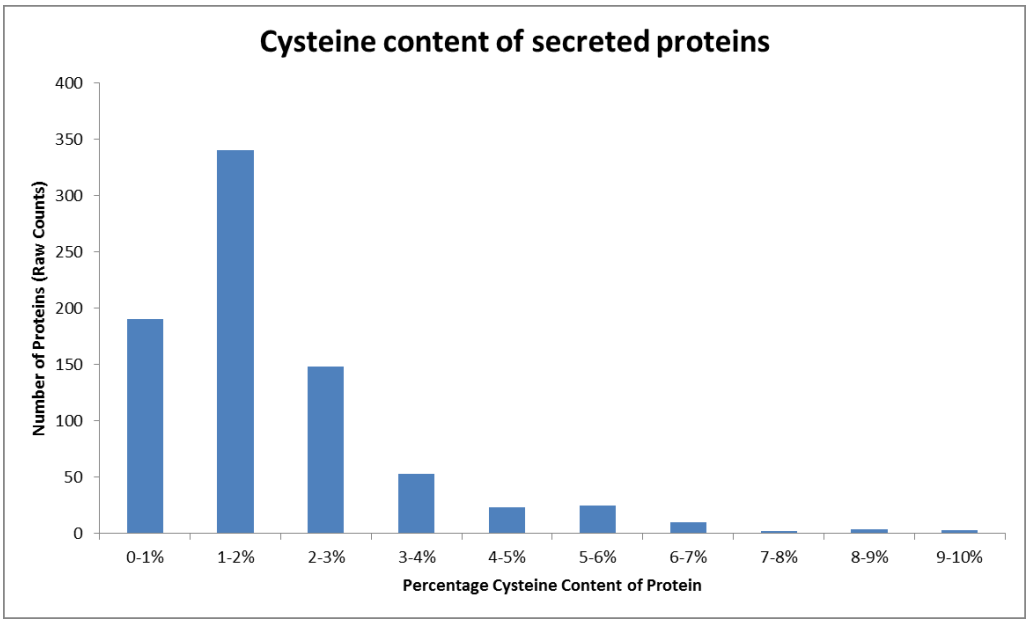


Figure 12: Percentage cysteine content of small secreted proteins. The majority of secreted proteins had between 0 and 3% cysteine content. Some had as high as 10%, though the majority of these were very small proteins such as hydrophobins.

4.4.3 Proteins with Known Effector Motifs

Domains reported to be involved in pathogenicity were detected in *Trichoderma* proteins (Saunders et al., 2012). These included CFEM (PF05730), an eight-cysteine-containing domain, present exclusively in fungi; proteins from the CAP protein family (**c**ysteine-rich secretory proteins, **a**ntigen 5, and **p**athogenesis-related 1 protein (CAP)) as well diverse protease inhibitors and LysM domain proteins were identified (**Supplementary Table 1**).

Manual and bioinformatic (R script) searches for known effector motifs revealed the presence of RxxL**,** RxxL-dEER motifs. WxxxE, KRKR and Lx2Rx4L motifs were also identified (**Supplementary Table 4**) within the three *Trichoderma* species, although some of these were found in small numbers likely to be accounted for by random chance. A KDxK motif was also found in 30 proteins often as a repeat sequence. ELM analysis suggests that KR rich motifs have a role in protein phosphorylation or localisation. They may therefore be relevant to effector movement or targeting in the host cell. It has previously been reported that KRKR motifs act as an NLS in bacterial effectors (Oliva et al., 2010). Eight proteins were found that had no homology to any known sequences, with a further 142 proteins having only one BLAST match, a full list of these is available in the supplementary material (**Supplementary Table 4**).

Several proteins with these motifs matched proteins of known function, such as transcription factors, protein kinases, protein phosphatases, Rho regulators, and proteins involved in the ubiquitination process (**Supplementary Table 5**). Proteins related to ubiquination, including zinc-fingers, F-box proteins and proteins involved in the three ubiquitin complexes. Ubiquination and phosphorylation are the most common post-translation modifications; both are involved in a number of cellular processes, including cellular differentiation and proliferation (Semple, RIKEN GER Group, & GSL Members, 2003). For a full list of effector proteins discussed in this chapter see **Supplementary tables 5 and 6**.

4.4.4 Cellular Localisation of Effectors

Protcomp analysis (Kundrotas & Alexov, 2007) identified the regions to which the secreted proteins are most likely localised. As effectors are assumed to be secreted by cross-referencing protein localisation signals to targets in the fungus and plant host, it was possible to identify putative effectors. Blast2Go results were used as a control e.g. known enzymes were excluded (**Supplementary Table 1**). Notable features identified were the large number of putatively secreted proteins that apparently localised, and remain bound to, the cell wall or plasma membrane. This could suggest a significant role for bound proteins in plant-fungal communication. Protcomp detected 1469 secreted proteins ('secretome'). However, only 422 were non-membrane bound. The number of proteins retained on the cell membrane was 857, with the remainder being plasma membrane bound.

4.4.5 Tandem Repeat Proteins

Proteins with tandem repeats, such as transcription activator–like (TAL) effectors, whose active regions are comprised of ~ 34 amino acid tandem repeats, may be important effector molecules. TAL effectors of plant pathogenic bacteria represent a new class of DNA-binding proteins (Scholze & Boch, 2010) which regulate gene expression in the host plant. TAL effectors are known to bind to DNA in the host cell in *Xanthomonas* (Boch & Bonas, 2010). Several tandem repeat proteins within the secretomes of the three *Trichoderma* spp. were identified (**Supplementary Table 3**). Tandem repeat proteins were from 65 to 1500 amino acids in length, with the majority of proteins being between 100-500 amino acids in length. They comprise 15.4% of the proteins identified as secreted in this study. The percentage of tandem repeats across the proteome is slightly lower at 12.4%. The

number of tandem repeats was remarkably similar between species, regardless of the smaller secretome size of *T. reesei*.

Repeat sequence proteins have potential to act as effector molecules, allowing delivery of multiple copies of an active domain to be transmitted into the host in a single protein. Several short repeat sites were identified (**Supplementary Table 3**) with the potential to serve as cleavage sites. Cleavage allows post-translational modification of the peptide into an active form (Müller, Schreier, & Uhrig, 2008). This has the potential to mask effectors until they are delivered into the target regions of the host, or to generate a higher copy number of an effector molecule. It may also allow targeting and regulation of effector molecules. Regardless of their purpose, the fact that 15.4% of the secretome is comprised of tandem repeat proteins suggests that these are worthy of further investigation. Several fungal cell wall proteins, including those responsible for chitin scavenging activity during plant colonisation (LysM), contain tandem repeats. In addition, some tandem repeat proteins, such as Msb2 in *U. maydis* (Lanver, Mendoza-Mendoza, Brachmann, & Kahmann, 2010) and *F. oxysporum* (Pérez-Nadales & Di Pietro, 2011), are involved in the perception of the host cell and in the transmission of plant signals required for successful plant colonisation.

4.4.6 Functions of Putative Effectors in *Trichoderma*

4.4.6.1 Protease Inhibitors

In oomycetes, three types of apoplastic effectors have been described: inhibitors of host enzymes, proteins involved in cell wall/plasma membrane adhesion and toxins that lead to host death (Senchou et al., 2004; Stassen & Van den Ackerveken, 2011). Additionally, in filamentous fungi there are proteins involved in the protection of hyphae from host attack (de Jonge et al., 2010; de Jonge & Thomma, 2009). Surprisingly, although protease inhibitors are common in oomycetes (between 18 to 43 different proteins) (Stassen & Van den Ackerveken, 2011) *Trichoderma* spp. contain only a few examples of these proteins. *Trichoderma atroviride* and *T. virens* have three putative protease inhibitors, whereas *T. reesei* contains only two (Table **14**).

The protease inhibitors mentioned above contain several known domains and are homologous to some known proteins. Three clear groups of protease inhibitors are identifiable in our data set. Group one (TR_111915, TV_92793 and TA_50405) all contain Kazal domains, group two (TV_10277

and TA_323283) contain IPR00037, IPR011052 and IPR018871 domains and group three (TV_177054, TA_300122) contains IPR009020 domains (**Supplementary Table 5**).

The Kazal domain of group one is a commonly found serine protease inhibitor domain that often occurs as a tandem repeat (Kamoun, 2007). These specifically inhibit S1 serine proteases by either lock or key or conformational change mechanisms. IPR00037 has previously been detected in squash and acts as a serine protease inhibitor (Hayashi et al., 1994). Other examples of proteins containing this domain have been identified as trypsin and elastase inhibitors . It is notable that these inhibitors have not previously been detected in fungi [http://www.ebi.ac.uk/interpro/IEntry?ac=IPR000737]. The second domain of group two, IPR0011052, is the active inhibitor domain and is also similar to carboxypeptase inhibitors. The final domain, IPR018871 is found in fungal adhesins which aid in attachment to cell surfaces. This suggests the inhibitor remains bound to the cell wall, which is further supported by the ProtComp localization analysis. This may indicate that these proteins have a protective role on the cell wall. The IPR009020 domain of group three is a protease propeptide inhibitor domain. These regulate protein folding and activity of peptidases in such a way as to block the substrate from the active site. These inhibitors are found in many organisms and can inhibit a wide range of enzymes.

The limited number of protease inhibitors detectable in these three *Trichoderma* species suggests that either protease inhibitors have a broad spectrum effect or inhibition of plant proteases is not a major mechanism behind *Trichoderma*'s ability to survive as a plant endophyte.

Table 14: Protease inhibitors in the three analysed *Trichoderma* strains

Seq. Identifier*	Seq. Description	Seq. Length	#Hits	min. eValue	InterPro Domains**
TA_300122	Proteinase propeptide	96	20	5.00E-65	IPR009020; GENE3D
TA_323283	Proteinase inhibitor I7	1238	19	0	IPR000737; IPR011052; IPR018871
TA_50405	Kazal domain-containing protein	149	20	1.46E-90	seg (SEG), SignalP-NN (euk) (SIGNALP)
TR_111915	Kazal domain-containing protein	143	20	4.51E-99	no IPS match
TR_72379	Proteinase inhibitor	893	17	0	IPR018871
TV_10277	Proteinase inhibitor I7	851	20	0	IPR000737; IPR011052; IPR018871
TV_177054	Proteinase propeptide	96	20	1.38E-63	IPR009020;GENE3D
TV_92793	Kazal domain-containing protein	143	20	2.46E-98	SignalP-NN (euk) (SIGNALP)

*Sequence identifiers (Seq. Identifier) show which species and which gene each description relates to. TA = *T. atroviride*, TV = *T. virens* and TR = *T. reesei*.

**Interpro domain codes .

4.4.6.2 Necrosis and Ethylene Inducing Peptides

Necrosis and ethylene inducing like peptides (NEP) and NEP-like are toxins identified in many different microorganisms including fungi, oomycetes and bacteria (Küfner, Ottmann, Oecking, & Nürnberger, 2009). NEPs elevate internal K^+, H^+ and Ca^{2+} levels, activate mitogen-activated protein kinases (MAPKs), and promote the accumulation of reactive oxygen species, and pathogenesis-related proteins, production of ethylene, callose deposition, and localised cell death (Küfner et al., 2009). Interestingly, these proteins seem to elicit necrosis and ethylene production only in dicotyledonous plants but are inactive in monocots (Bae, Bowers, Tooley, & Bailey, 2005; Bae, Kim, Sicher, Bae, & Bailey, 2006; Ottmann et al., 2009). However, NEP-like encoding genes are also present in pathogens of monocots that do not elicit necrosis, suggesting additional roles for these of proteins (Motteram et al., 2009). Despite their diverse distribution across taxa, most of the NPLs share a common fold characterised by a heptapeptide (GHRHDWE) motif (Bae et al., 2005, 2006; Ottmann et al., 2009), although it has been shown that only 36% (12 over 33 proteins) of NEP-like proteins from *Phytophthora* possess a full complement of the amino acid residues from this domain (Dong et al., 2012). *Trichoderma* spp. have four encoding genes containing a putative NEP-like protein domain (IPR008701) (**Table 15**); nevertheless although they have variations in the heptapeptide sequence, the proteins contain the amino acids residues required for the full activity of NEPs reported in other fungi (Kleemann, 2010).

Table 15: Necrosis and ethylene inducing peptides in the three analysed *Trichoderma* strains

Seq. Identifier	Seq. Description	Seq. Length	#Hits	min. eValue	InterPro Domains**
TV_46830	Necrosis inducing protein (NPP1)	390	20	1.90E-161	IPR008701
TA_302472	Necrosis inducing protein (NPP1)	282	20	0	IPR008701
TV_52963	Necrosis inducing protein (NPP1)	245	20	2.30E-05	IPR008701
TV_50664	Necrosis inducing protein (NPP1)	253	20	2.03E-07	IPR008701

Sequence identifiers (Seq. Identifier) show which species and which gene each description relates to. TA = *T. atroviride*, TV = *T. virens* and TR = *T. reesei.* ***Interpro domains (http://www.ebi.ac.uk/interpro/).

Trichoderma virens contains three NEP-like proteins: TV_46830 has a significant E-value of 1.9E-61, while TV_52963 and TV_50664 have lower values (2.30E-05 and 3.20E-07, respectively). Although these two latest proteins share identity to NEP-like proteins, they lack a signal peptide. This could be due to improper annotation (TV_52963) of the genome of *T. virens,* or due to differences in the secretion mechanisms (TV_50664) in *Trichoderma,* as has previously been suggested for other

effectors from filamentous pathogens (Kamoun, 2006; Oliva et al., 2010). NEP like proteins are proteins that usually possess an N-terminal secretion signal peptide followed by a semi-conserved domain, called necrosis and ethylene inducing peptide (Dong et al., 2012; Küfner et al., 2009). The proteins TV_46830 and XP_001804225.1 have an additional domain between the signal peptide and the NEP domain, which is unrelated to other proteins or domains. It was observed that NEP-like proteins share their conserved domain to acyl thio-esterases suggesting other roles for these proteins. Several NEP-like toxins from *Phytophthora sojae* lack necrosis-inducing activity which supports this suggestion (Dong et al., 2012).

4.4.6.3 Pathogenesis-related (PR) Proteins: Thaumatin-like Proteins (TLP)

Thaumatin like proteins (IPR001938) are pathogenesis-related (PR) proteins of family 5 (Chu & Ng, 2003). They form a group of proteins with proprieties such as antifungal activity (Chu & Ng, 2003). TLPs are induced in plants in response to various biotic and abiotic stresses and have been assumed to play a role in plant defence systems, particularly in SAR (Cornelissen, Hooft van Huijsduijnen, & Bol, 1986). Many TLPs accumulate in plants in response to infection by a pathogen and possess antifungal activity (Chu & Ng, 2003; Ho, Wong, & Ng, 2007; Leone et al., 2006; Vitali et al., 2006; H. Wang & Ng, 2002). Some TL proteins hydrolysed crude fungal walls and one barley TL enzyme even lyses fungal spores (Cornelissen et al., 1986). TL proteins are present in the tree species of *Trichoderma* analysed here, but a secretion signal was found only in *T. atroviride* (TA_ 306146, TA_224184, TA_32968) and in *T. virens* (TV_65931, TV_70385) proteins but not in the saprobic fungus *T. reesei* (TR_109835).

Xylanase inhibitor proteins (XIP) are considered potential defence molecules, which prevent cell wall degradation by hydrolytic fungal enzymes (Leone et al., 2006). The presence of thaumatin-like xylanase inhibitors (TLX) and chitinase-like xylanase inhibitors proteins was recently reported in plants (Ho et al., 2007; Vasconcelos et al., 2011).This opens up the possibility that TLP from *Trichoderma* could act as potential effector proteins by inhibiting plant chitinases that are activated during root colonisation.

4.4.6.4 Trichoderma LysM-like Effectors

Chitin is an N-acetyl-D-glucosamine (GlcNac) homopolymer found as a primary structural component in the cell wall of fungi (Kombrink, Sánchez-Vallet, & Thomma, 2011). As plants do not contain chitin, this molecule is recognised as a non-self-component and activates host immunity (de Jonge et al., 2010; de Jonge & Thomma, 2009; Marshall et al., 2011). In fungi, several effector proteins that prevent immune activation in response to the release (via degradation by host attack) of fungal cell wall chitin have been identified. These effectors contain diverse number of LysM domains (IPR002482) that bind chitin. A lectin-like LysM protein from *Cladosporium fulvum* was found to inhibit chitin oligosaccharide-triggered and PRR-mediated activation of host immunity in tomato (de Jonge et al., 2010).

Table 16: Thaumatin pathogenesis proteins in the three analysed *Trichoderma* species

Seq. Identifier	Seq. Description	Seq. Length	#Hits	min. eValue	InterPro Domains**
TA_ 306146	Thaumatin family protein	253	8	0	IPR001938
TA_224184	Thaumatin	270	8	0	IPR001938
TA_32968	Thaumatin	246	6	1.65E-166	IPR001938
TA_233988	Thaumatin	242	-	-	-
TR_ 109835	Thaumatin family protein	355	20	0	IPR001938
TV_65931	Thaumatin family protein	496	20	0	IPR001938
TV_70385	Thaumatin	246	6	2.47E-166	IPR001938
TV_213200	Thaumatin	268	8	0	IPR001938

* Sequence identifiers (Seq. Identifier) show which species and which gene each description relates to. TA = *T. atroviride*, TV = *T. virens* and TR = *T. reesei*. **Interpro domains were examined at http://www.ebi.ac.uk/interpro/.

Similar strategies have been observed in *Mycosphaerella graminicola* (Marshall et al., 2011) and *Magnaporthe oryzae* (Mentlak et al., 2012). LysM proteins are thought to operate by scavenging or recruitment of chitin molecules that have been released, thus preventing host detection. De Jonge and Thomma (2009) suggested that *T. atroviride* and *T. virens* contain 10 potential LysM effectors each and that *T. reesei* only has three (de Jonge & Thomma, 2009). In this analysis on only four LysM potential effectors were identified in *T. atroviride* (TA_297859, TA_307020, TA_43321, TA_85797), four in *T. virens* (TV_124493, TV_128337, TV_149422, TV_66683) and two in *T. reesei* (TR_105336 and TR_54723).

Table 17: LysM domain proteins identified in the three analysed *Trichoderma* strains.

Seq. Identifier*	Seq. Description	Seq. Length	#Hits	min. eValue	InterPro Domains**
TA_297859	LysM domain- GPI-anchored	746	20	0	IPR002482; IPR018392
TA_307020	LysM domain	688	20	0	IPR001002; IPR018392
TA_43321	LysM domain	511	20	0	IPR002482; IPR018392
TA_85797	LysM domain- GPI-anchored	544	20	0	IPR002482; IPR018392
TR_105336	LysM domain	473	20	0	IPR002482; IPR018392
TR_54723	LysM domain	276	20	4.73E-156	IPR002482; IPR018392
TV_124493	LysM domain- GPI-anchored	646	20	0	IPR002482; IPR018392
TV_128337	LysM domain- GPI-anchored	356	20	8.17E-172	IPR002482; IPR018392
TV_149422	LysM domain- GPI-anchored	782	20	0	IPR002482; IPR018392
TV_66683	LysM domain- GPI-anchored	319	20	2.53E-175	IPR002482; IPR018392

*Sequence identifiers (Seq. Identifier) show which species and which gene each description relates to. TA = *T. atroviride*, TV = *T. virens* and TR = *T. reesei*. **Interpro domains can be examined here http://www.ebi.ac.uk/interpro/.

4.5 Other Secreted molecules

Although the primary focus of this study was identification of putative effector molecules, a number of major non-effector protein groups were also detected by bioinformatics analysis. It should however be noted that these proteins have been deemed effectors in some parts of the literature, and it is possible that their other functions do include some signalling and effector activity, though this may not be their primary function (Kubicek, Starr, & Glass, 2014). These molecules include proteases, cell wall degrading enzymes (CWDE) and oxidation-reduction pathway proteins such as cytochromes. While these proteins are not effector molecules due to the lack of direct interaction with the host plant, they are likely to play a role in root colonisation via alternative mechanisms. Serine proteases and subtilisin like proteases are known to degrade a range of proteins, potentially disrupting maize responses to the invading organism or enhancing fungal penetration of the host cell(Lindstrom, Sun, & Belanger, 1993; Oh et al., 2008; Reddy, Lam, & Belanger, 1996). Thirteen serine proteases and eleven subtilisin-like proteases were detected amongst the three strains studied. Cell wall degrading enzymes have been detected in mutualistic interactions in *P. indica* and may enable fungal penetration and feeding (Moy, Li, Sullivan, White, & Belanger, 2002; Zuccaro et al., 2011). Alternatively these proteins may be part of a signalling/sensing mechanism related to the release of degraded cell wall products (Zuccaro et al., 2011). It is difficult to identify the primary function of these from a bioinformatics study, but chitinases, xylanases, cellulases and a range of other polysaccharide degrading enzymes were identified in the secretome dataset, which would be likely to enable either behaviour in these three *Trichoderma* species. Cytochromes are linked to

oxidation reduction as well as diverse biosynthetic pathways. Secondary metabolites often have major regulatory roles in plants and are produced by *Trichoderma* spp. (Lah et al., 2011; Mukherjee, Buensanteai, Moran-Diez, Druzhinina, & Kenerley, 2012). Cytochromes have also been linked to survival in low oxygen environments in some bacterial endophytes (Colburn-Clifford & Allen, 2010).

4.6 Conclusion

Trichoderma species possess a large array of secreted proteins that allow them to partake in diverse lifestyles. Around 6% of the proteome is composed of secreted proteins that are potential effectors. Intriguingly however, previous studies have shown that the differences in secretome size are not necessarily accountable for the endophytic or pathogenic behaviour of effector possessing species. A number of non-effector proteins that may be relevant to root colonisation were identified which may be worthy of further investigation. Finally, numerous *Trichoderma* proteins show no homology to any protein in the BLAST database. The low homology to known proteins is common to effector proteins and these are potentially good targets for future study into the endophytic behaviour of *Trichoderma* spp.

5 Transcriptome Profiles of Interacting *T. virens and Zea mays* Roots Indicate Polysaccharide Catabolism and Secondary Metabolism as the Primary Response Induced

5.1 Abstract

Illumina Hi-Seq is a method for the determination of transcript counts using RNA sequence analysis. This study used next generation sequencing to measure differentially expressed transcript counts in a *Trichoderma virens* and *Zea mays* interaction system. These were compared to both the fungus grown in isolation on media and control plants grown in soil. Total RNA was isolated from the top 2 cm root segments of maize plants, containing endophytic *Trichoderma*, at various times post inoculation, from 12 h to 7 d. Total RNA was sequenced using the Illumina Hi-Seq 2000 system. Differential expression analysis was carried out using EdgeR and the CLC Genomics workbench. This experiment aimed to determine if predicted effector genes were expressed in the interaction and whether expression of these genes was consistent across time points. This analysis demonstrated a consistent up regulation of unknown proteins, glycoside hydrolase enzymes and sugar transporters at all times post inoculation. This suggests that the unknown protein set is likely to contain effector proteins of novel function and that a polysaccharide degradation pathway is a major part of the interaction between these two organisms. Enzymes are most likely required to degrade plant cell wall (which is composed of several polysaccharides targeted by these enzymes). This may be able to activate plant damage recognition pathways during fungal colonisation. This is supported by the up regulation of a number of classical plant recognition receptors as well as two inhibitors of cell wall degrading enzymes.

5.2 Introduction

Advances in nucleotide sequencing (collectively known as next generation sequencing or NGS) technology have enabled affordable high throughput sequencing on a scale sufficient for comparative transcriptome analysis. Illumina HiSeq is one of the predominant NGS platforms currently in use and is capable of generating stranded (directional) paired reads up to 100 bases in length (Illumina Hi-Seq 2000 Specifications). Approximately 200 Gigabases (Gb) of data can be generated per run, enabling high-resolution analysis of the target organism. Illumina NGS technology has been used to identify and measure transcription of novel genes and non-coding RNAs in a variety of organisms (Alarcón, Lee, Goodarzi, Halberg, & Tavazoie, 2015; Ayala, Wang, Benitez, & Silva, 2015; Mayer et al., 2015). Application of this study to plant-pathogen interactions is now common, with published transcriptomes available for a variety of fungal species, such as *Epichloe*, *Fusarium* and *Magnaporthe*, in interaction with their plant hosts (Eaton et al., 2014; Lysøe, Seong, & Kistler, 2011; Oh et al., 2008). NGS has only recently been applied to endophytic systems (Morán-Diez et al., 2015; Zuccaro et al., 2011). In this study molecular communication during endophytic interactions was examined using Illumina HiSeq technology, in a model system comprising *Trichoderma virens* and maize roots (*Zea mays*).

Trichoderma spp. are generally known for their capacity to use a wide variety of substrates, allowing them to survive in a large variety of habitats and lifestyles. The capacity to digest a broad range of substrates is conferred by the large number of proteins and secondary metabolites these fungi can produce. *Trichoderma reesei* is widely used in industry to produce lytic enzymes, yet it is considerably less diverse in functionality and more limited in lifestyle than its relatives *Trichoderma atroviride* and *Trichoderma virens* (See chapter one) (Peterson & Nevalainen, 2012). **Figure 13** shows a detailed breakdown of predicted *T. virens* protein functions (from **Chapter 4** data), which is important in comparing the relative abundance of differentially expressed genes to the amount existing in the genome of *T. virens*.

Only a limited number of these products are predicted to be relevant to the endophytic lifestyle of *Trichoderma* spp. However, recent evidence suggests that endophytic interactions may share several mechanisms with pathogens; albeit with a different outcome for the host plant. Effector proteins are likely to play a role in endophytic interactions. Around 60 effectors have been identified amongst fungal systems, while considerably more is known about bacterial effectors

(Sperschneider et al., 2015). Approximately 500 effectors are known, with 39 being demonstrated in *E. coli* alone (Burstein et al., 2009; Dean & Kenny, 2009; Tobe et al., 2006; Van Engelenburg & Palmer, 2010). The diversity of effector proteins and their low homology to known proteins can make them difficult to predict. Bioinformatic predictions suggested that approximately half of *T. virens* proteins with effector-like characteristics were of unknown function (see **Chapter 4**). Therefore it is hypothesised that proteins of unknown function will form a major set of differentially expressed proteins, and that a subset of these will be effectors.

Effector delivery systems are likely to be up-regulated during the interaction if effectors proteins are present. Bacterial effectors tend to be delivered by either the type II (arginine translocation) or the type III secretion system (T3SS – Calcium gated 'syringe') (Barnett, Eijlander, Kuipers, & Robinson, 2008; Dean, 2011; Sargent, Berks, & Palmer, 2006). However it is known that six bacterial secretion systems exist (Costa et al., 2015; Tseng, Tyler, & Setubal, 2009). Fungal effectors are delivered by as yet unknown methods, however several known effectors follow the classical secretion signalling pathway (Endoplasmic reticulum-Golgi Pathway) (Harman et al., 2004b) . Recent evidence suggests that cytoplasmic effectors are primarily secreted via this ER-Golgi system, but effectors localised to the interior of host cells follow separate mechanisms (Giraldo et al., 2013). A number of other secretion systems exist; in *Magnaporthe* formation of a plant-membrane biotrophic interfacial complex (BIC) allows secretion of effectors into the plant by invasive hyphae (Giraldo et al., 2013; S. Zhang & Xu, 2014). Export of proteins into the BIC appears to involve a SNARE system, which has also been observed as a secretion system operating in *T. reesei*, which can localise export of molecules to various hyphal regions (Giraldo et al., 2013; Valkonen et al., 2007). Haustorium type feeding structures and various morphological features such as appressoria and invasive hyphae are also implicated in effector delivery, though subsequent translocation is still thought to rely on a variety of protein secretion signals (Bozkurt, Schornack, Banfield, & Kamoun, 2012; Petre & Kamoun, 2014; Rafiqi, Ellis, Ludowici, Hardham, & Dodds, 2012). Genes related to transport, haustorium formation and effector delivery may therefore be differentially expressed.

Cell wall degrading enzymes (CWDE) such as hydrolases are a major group involved in pathogenesis and they make up a considerable portion of the enzymes identified in *T. virens* (**Figure 13**) (Abubaker, Sjaarda, & Castle, 2013; Gilbert, 2010). Glycoside, anhydride and ester hydrolases made up a large section of the *T. virens* proteome. Anhydride hydrolases include proteins such as GTPases which are commonly involved in signal transduction or cellular regulation and are known to

regulate major processes such as conidiation in *Trichoderma* (Rocha-Ramírez, Omero, Chet, Horwitz, & Herrera-Estrella, 2002). Ester hydrolases have been predominantly studied for their uses in the industrial degradation of polyesters, however it has been observed that esterification of the plant cell wall can play a role in protecting it from hydrolysis by other lytic enzymes (Lionetti, Cervone, & Bellincampi, 2012). Glycoside hydrolases form a diverse group of polysaccharide degrading proteins, of which 113 families are known. Each family tends to be linked to a specific function; those most relevant to pathogenesis are the cellulases, pectinases and xylanases, all of which allow degradation of the plant cell wall (Bellincampi et al., 2014; Gilbert, 2010). In *Trichoderma* these have been linked to saprotrophic feeding on plant hosts. Degradation products from plant cell wall polysaccharides have been shown to be a component of plant defence signalling, with sucrose, hexose and trehalose being identified as signalling polysaccharides (Talbot, 2010; Wahl, Wippel, Goos, Kämper, & Sauer, 2010). Polysaccharide transport proteins are therefore likely to play a role both in delivery of effector polysaccharides and in the recovery of various substrates for signalling or nutrition, such as monosaccharide subunits of polysaccharides, released by degradation of various moieties. Due to their potential roles in cell wall penetration, communication and nutrition, CWDE's and sugar transporters may form a major up-regulated group in *T. virens*-maize interactions.

Peptidases, such as serine proteases form a second group of lytic enzymes produced by *Trichoderma*. These are responsible for the cleavage and degradation of other proteins. In pathogenic systems these proteins are responsible for targeted degradation of plant defence proteins. The plant will also secrete these to attempt to degrade fungal proteins and inhibit colonisation. Subtilisin-like serine proteases are crucial to penetration and growth within the host in *Magnaporthe oryzae* (Oh et al., 2008). Inhibitors of these enzymes are also likely to play a role in root colonisation, by preventing activity of these lytic enzymes. Enzyme inhibitors may also be responsible for preventing programmed cell death in the plant host, which is a common defensive response to root colonisation by pathogenic fungi (Dickman & de Figueiredo, 2013).

Volatile compounds such as plant hormones, steroids and various secondary metabolites have long been known to affect plant behavior (Naznin et al., 2014). Plant hormones, such as jasmonic acid (JA), Gibberellins and salicylic acid (SA) can modulate plant defence pathways resulting in systemic defence responses (Halim et al., 2006; Sun, 2010). These mediate responses to colonisation by

regulating plant processes such as the oxidative burst and can also affect production of phenolic compounds by the plant (War, Paulraj, War, & Ignacimuthu, 2011).

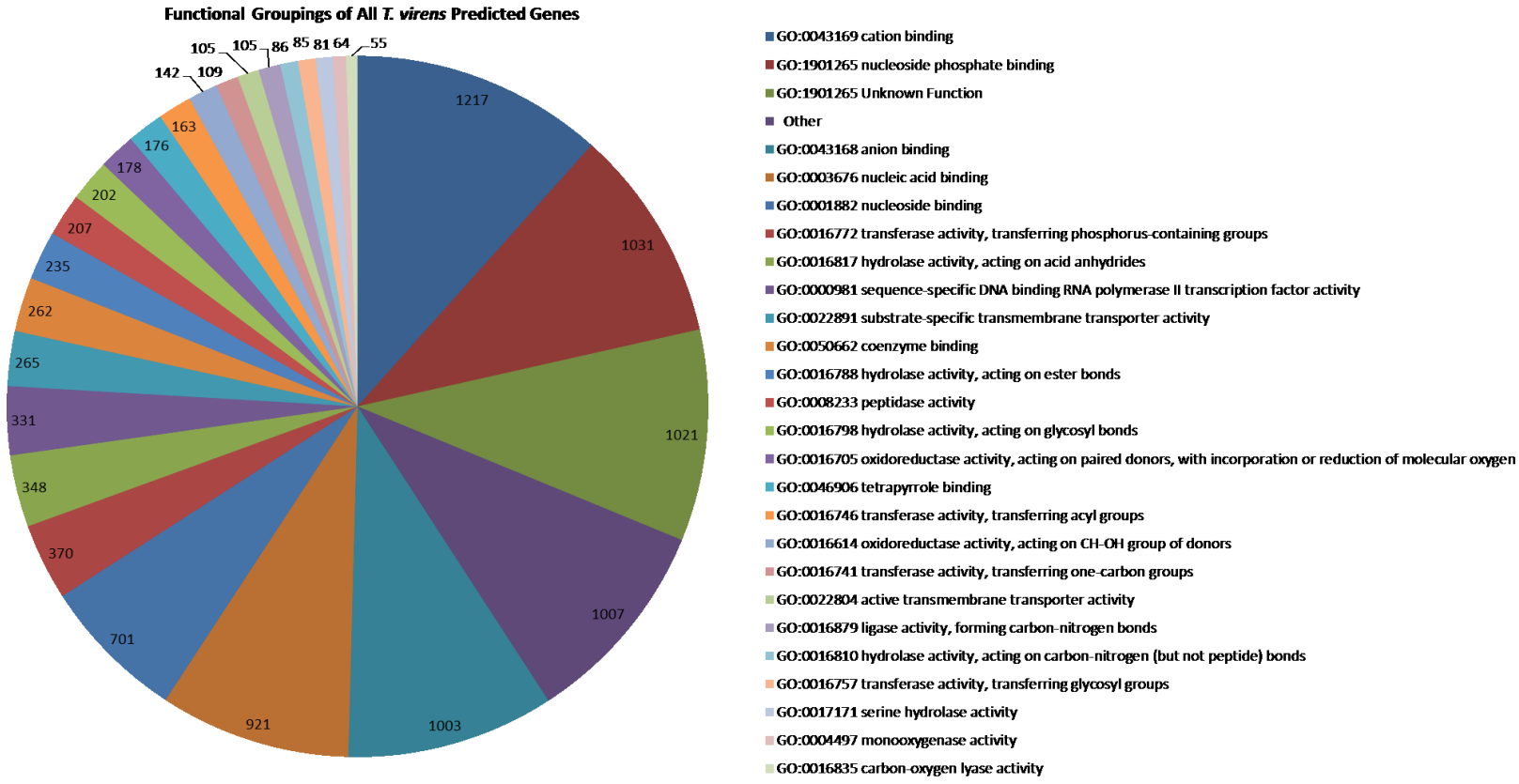


Figure 13: Functional categories for genes comprising the entire proteome of *T. virens*. BLAST, Interpro and GO searches implemented in BLAST2GO were used to construct functional categories for all of the 12427 genes present in *T. virens*. The number of proteins within each category is labelled inside the pie slices. This serves as a reference point for comparison to later functional diagrams as it demonstrates the relative abundance of proteins within a functional group compared to the number of such proteins that are differentially expressed. Glycoside hydrolases may also elicit a plant immune response through the release of Damage Associated Molecular Patterns (DAMP), which responds to degradation products of cell wall polysaccharides (Ma, Zhao, Walker, & Berkowitz, 2013).

Compounds of this type may be produced by the fungus to interfere with plant immune signalling. The ability of fungi to promote growth in plants can also be mediated by these compounds, particularly where ethylene signalling is involved (Khatabi et al., 2012). The jasmonic acid/ethylene pathway in *Trichoderma asperellum* is responsible for enhancing plant immunity to other pathogens (Shoresh, Yedidia, & Chet, 2005). The shikimate biosynthetic pathway is responsible for precursors to many of these compounds and regulatory changes and differential expression of these genes would suggest a functional role for volatile compounds in root colonisation.

Cation and Anion binding functionalities form a major component of *T. virens* proteome (**Figure 13**) however it is difficult to elicit the exact function of this diverse group. Heme centred molecules such as siderophores may be relevant to root colonisation as a mechanism of iron scavenging from the plant host (Hao, Willis, Andrews-Polymenis, McClelland, & Barak, 2012). Metal ions are also likely to be involved in redox responses, perhaps as a mechanism of defence against plant oxidative bursts and fungal iron scavenging ability has been linked to a reduction in virulence in some fungal pathogens (Greenshields, Liu, & Wei, 2007). Monooxygenases, such as cytochromes, have been shown to be responsible for fungal resistance to plant volatiles such as phenols. Some of these are also involved in volatile biosynthetic pathways in the fungus (Lah et al., 2011). Zinc based proteins, such as zinc finger proteins, are commonly involved in defence signalling in plant resistance (R) proteins and in transcriptional regulation in fungi (Gupta, Rai, Kanwar, & Sharma, 2012; MacPherson, Larochelle, & Turcotte, 2006). These proteins are highly likely to be involved in the communication between maize and *T. virens*.

The endophytic lifestyle presents a number of unique challenges to *T. virens* that require a concerted but well balanced response, which is likely to be highly complex and variable over time. Initial endophytic interactions have been reported as early as 12h with longer term studies lasting periods of weeks, although generally not under sterile conditions (Brotman et al., 2013; Mukherjee, Horwitz, Singh, Mukherjee, & Schmoll, 2013). As this study is focused on root colonisation total RNA from early colonisation points were taken, in a range from 12 h post inoculation to 7 days post inoculation. NGS technology is ideally suited to the scope of this experiment, allowing a broad spectrum analysis of expression across each of the selected interaction periods. Using differential expression analysis this study aims to identify reproducible patterns in the *T. virens*-maize interactions and examine their potential effects on pathways linked to fungal colonisation.

5.3 Methods

5.3.1 Seed sterilisation and Plant Growth

Maize hybrid line 34H31 was used for this experiment as a result of the experiments in **Chapter 2**. Seeds were sterilised according to the method in **section 2.6.7.1**. Plants were grown according to the sterile soil growth method (**section 2.6.7.2**). Four control maize plants were planted for each time point, planting was performed immediately after seed sterilisation. Control plants were then sealed into Systema breakfast cereal containers. Experimental maize seeds were inoculated with 1 million spores prior to planting and left to dry for 30 min in a sterile cabinet. Sixty inoclulated maize plants were planted, 12 samples were taken by random selection for each time point (e.g. 12 plants at 3 DPI, 12 at 5 DPI and 12 and 7 DPI). Time points were selected based on microscopic data from **Chapter 3** and from prior experience with pilot studies at 12, 24 and 48h post inoculation (see **Appendix I**).

5.3.2 Inoculation

Three controls of *T.* virens grown *in vitro* were prepared, by inoculation of 1 million spores onto a 20 ml plate of minimal media (**see Table 3**) covered with cellophane. Spores were then allowed to grow for 7 d at 25°C before harvesting.

5.3.3 Total RNA Isolation

Trichoderma virens was harvested by gently removing the mycelia from the cellophane and then snap freezing and grinding it under liquid nitrogen. These were used to compare expression on *in vitro* growth media to expression in endophytic growth.

Maize plants were destructively harvested under liquid nitrogen at 3, 5 and 7 d. In **Chapter 3** it was determined that fungal material was most commonly present in the top 2 cm of the root. Therefore top 2cm of each root was sectioned and ground under liquid nitrogen. Twelve plants were harvested at each time point. Root sections from four randomly selected maize plants were pooled to form each sample. This resulted in three samples for each time point, comprised of four pooled plant roots for each time point (**Table 9**).

RNA was extracted according to **section 2.6.2**. Total RNA samples were quantified using the Nanodrop and their integrity was verified using 0.8% Agarose gel electrophoresis performed at 110 V and 500 mA for 25 min.

5.3.4 Illumina Sequencing

cDNA libraries were prepared according to Illumina® instructions by the New Zealand Genomics Lab (NZGL). Total RNA was sequenced on an Illumina® HiSeq™ 2000 sequencing system (Bentley et al., 2008). Twenty four samples were sequenced on a sequencing lane with 100 base paired directional (stranded) reads.

Table 18: DPI samples run on Illumina HiSeq

Samples Run on Illumina Hi-Seq	Quantity of RNA (ng)
Maize Control 3 DPI	1647.36
Maize Control 5 DPI	1035.6
Maize Control 7 DPI	1224.48
T. virens inoculated maize 3 DPI #1	1952.4
T. virens inoculated maize 3 DPI #2	2352.15
T. virens inoculated maize 3 DPI #3	1819.8
T. virens inoculated maize 5 DPI #1	1666.5
T. virens inoculated maize 5 DPI #2	1663.65
T. virens inoculated maize 5 DPI #3	1882.5
T. virens inoculated maize 7 DPI #1	1644.9
T. virens inoculated maize 7 DPI #2	2231.1
T. virens inoculated maize 7 DPI #3	2388

Samples run on an Illumina HiSeq lane for the DPI experiment. The remaining 9 samples were for a separate experiment. 1000 ng of RNA is required for sequencing and the quantities sequenced are shown here. Descriptions identify the type of RNA and conditions it was extracted under.

5.3.5 Analysis of Read Data

Fastq output files from the Illumina® HiSeq™ were analysed with FastQC to confirm sequence quality. Sequences with a quality score under 20 were discarded. Low quality sequences and remaining adaptor sequences were removed using Trimmomatic set to paired end mode (Bolger, Lohse, & Usadel, 2014). *T. virens* sequences were mapped to the publicly available genome from the Joint Genome Institute (JGI, http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.info.html). Maize sequences were mapped to the B73 genome from MaizeGDB (http://www.maizegdb.org/). Mapping was performed initially with Tophat 2 (Kim et al., 2013), however due to problems with mapping to the maize genome (due to compatibility issues with the maize gff file), sequences were

subsequently mapped with CLC Genomics workbench (http://www.clcbio.com/products/clc-genomics-workbench/). The RNA-Seq toolkit (implemented in CLC genomics workbench) was used with the default settings (Mismatch: cost 2, Insert: Cost 2, Delete Cost: 3, Length fraction 0.8, Similarity fraction 0.8, Max hits/read: 10). The reproducibility of the data was assessed by measuring the biological coefficient of variation as calculated by EdgeR using the equation shown:

$$V^2(\gamma_{gi}) = \frac{1}{\mu_{gi}} + \varphi_g$$

Read counts from CLC were input into EdgeR (Robinson, McCarthy, & Robinson, 2010) implemented in R (http://www.r-project.org/) for differential expression analysis (See supplementary material for scripts). Sequences with less than a ±2 $\log_{10}$ change or a FDR (false discovery rate) value under 0.05 were discarded. To visualise data volcano plots, MA plots and all statistical analysis were performed using the EdgeR package. Heat maps were drawn by converting count data to FPKM (Fragments per Kilobase of exon per Million fragments) with Trinity (Haas et al., 2013) scripts. Transcripts with similar FPKM scores were then clustered using the Euclidean distance method. The pHeatmap R package (https://cran.r-project.org/web/packages/pheatmap/index.html) was then used to visualise the data. For detailed information on the scripts used see **Supplementary Document 1**. A diagrammatic explanation of the workflow is shown in **Figure 14**.

5.3.6 Functional Analysis

Differentially expressed genes were analysed for matches to known proteins. The entire *in silico* predicted proteome of *T. virens* was run in BLAST, using BLAST2GO (default settings) to automate the analysis (Settings: Blastp, nr Database, E value: 1.0e-3, 20 blast hits) (Conesa, 2005; Conesa & Gotz, 2008; Götz et al., 2008). Interpro scans and Gene ontology (GO) analysis were performed on these proteins to provide further functional details, particularly on proteins with no BLAST hits (Settings: E value: 1.0e-6, Annotation cut off: 55, GO weight: 5, Hsp hit coverage cutoff: 0, no taxonomic filter) (Zdobnov & Apweiler, 2001). Interproscan uses a large number of databases to look for potential motifs, including but not limited to TMMHMM, HMM, Blastprodom, Coils. Gene3D, Phobius and SignalP. The same analysis was performed on maize, however only differentially expressed sequences were analysed due to the size of the maize proteome. For practical reasons, graphs were drawn using the BLAST2GO combined graph function, using gene ontology level 4 (graphs became difficult to visualise below this level). Functionalities with less than 5 different proteins were grouped as other for purposes of visualization. Data was then analysed

manually and using Mapman to link functionalities to biochemical pathways (Thimm et al., 2004; Usadel et al., 2009). The top 50 most differentially up and down-regulated genes were identified in each dataset and heat plots were drawn from this data.

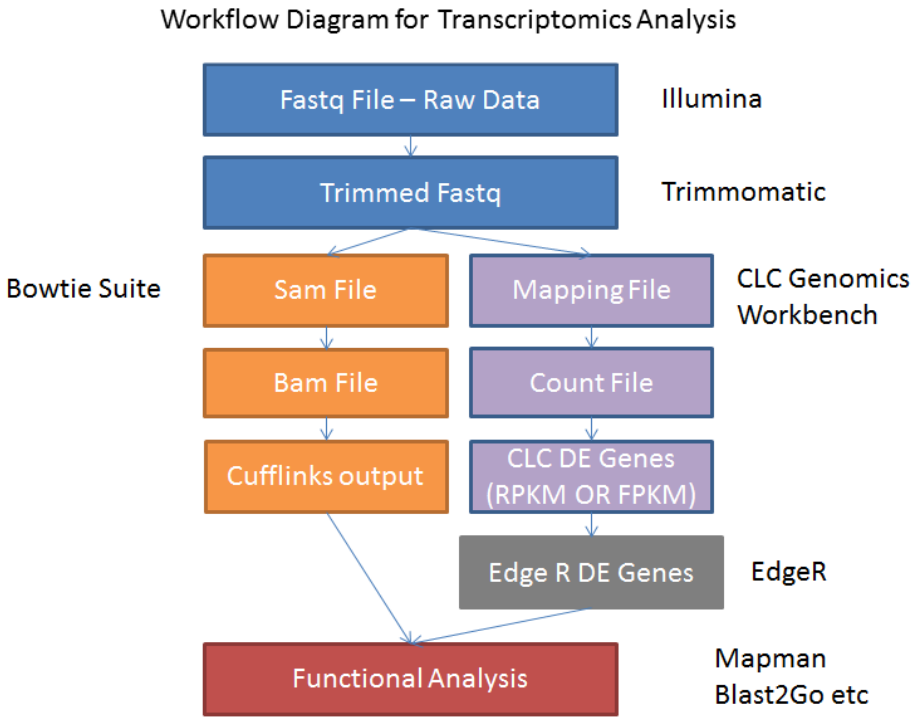


Figure 14: A diagrammatic outline of the workflow used in transcriptome analysis. Fastq files are the Illumina sequencing output file. CLC uses proprietary files. The bowtie suite works with sam and bam files. Differentially expressed (DE) genes are found by CLC, cufflinks and EdgeR. Functional analysis used a variety of programs including Mapman and BLAST2GO.

5.4 Results and Discussion: Maize-*T. virens* Interaction at Three, Five and Seven Days Post Inoculation

This experiment examined the transcriptional profile of *T. virens* and maize during root colonisation at 3, 5 and 7 days post inoculation. These periods of time were chosen to attempt to identify changes in transcription from the early to late stages of colonisation. This aimed to identify the longer lasting effects of root colonisation and identify molecules responsible for maintenance rather than initiation of the endophytic interaction. From herein these samples will be referred to as the Days Post Inoculation (DPI) dataset.

5.4.1 Quality Control – *T. virens* DPI Dataset

Read counts for the long *Trichoderma* time points were adequate for differential expression analysis and appeared reliable according to dispersion and BCV data (**Table 19**). All DPI datasets had an average per base quality score of 30 (32-40, mean 36) or more across the length of the read (100bp), with the exception of positions 50-54 which showed a drop in quality (scoring between 14-32, mean 22) (**See Supplementary Material 'FastQC – DPI'**). These positions are almost directly in the centre of the read and are unlikely to affect read mapping (**Figure 15**). BCV and dispersion values are low, indicating good replication of the data across samples. This is reflected in the high resolving power and number of significantly differentially expressed genes detected, with a low number of outlier genes (**Figure 16**).

Table 19: Reads mapping to *T. virens* - DPI Experiments

Sample	*T. virens* reads	Dispersion	BCV
3 DPI 1	1110456	0.09034	0.3006
3 DPI 2	816037		
3 DPI 3	303399		
5 DPI 1	723744	0.1448	0.3805
5 DPI 2	885271		
5 DPI 3	811318		
7 DPI 1	501316	0.13497	0.3674
7 DPI 2	598970		
7 DPI 3	470794		

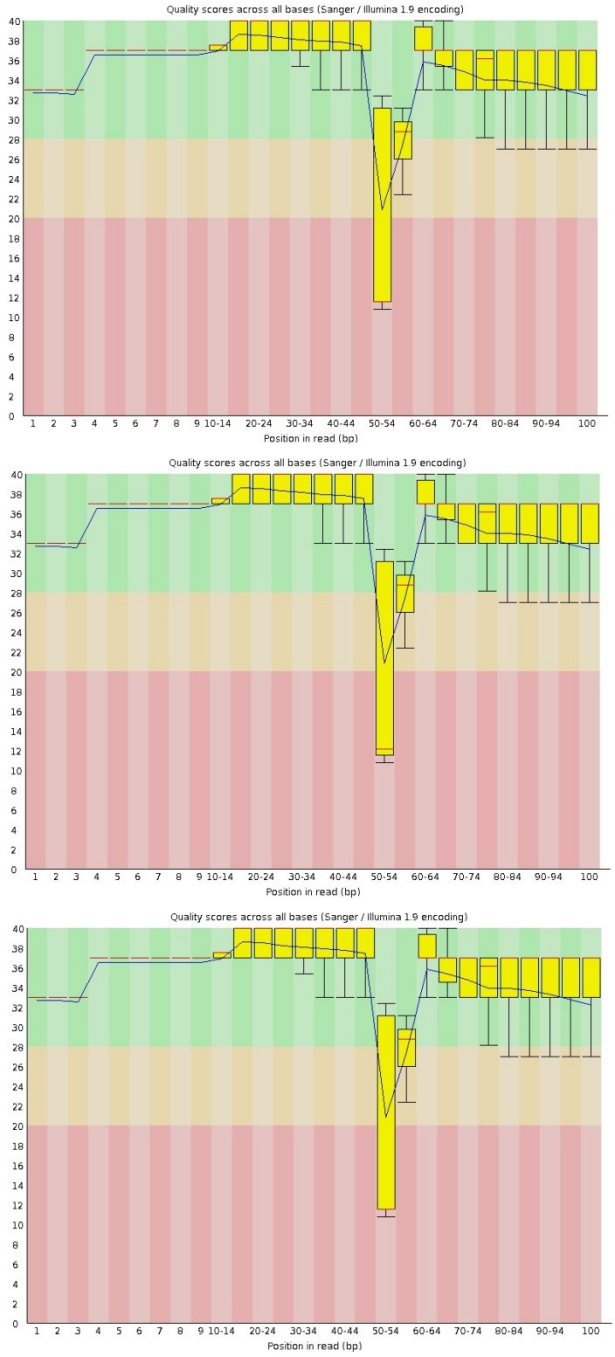


Figure 15: FastQC quality scores for 3 DPI 1 (A), 5 DPI 1 (B) and 7 DPI 1 (C). This graph is representative of the results obtained for all of the DPI samples. Read quality is high with the sole exception of the region at 50-54 bp along the read. As sequence mapping relies more on the ends of the read being correct, this is unlikely to dramatically affect the transcriptome results.

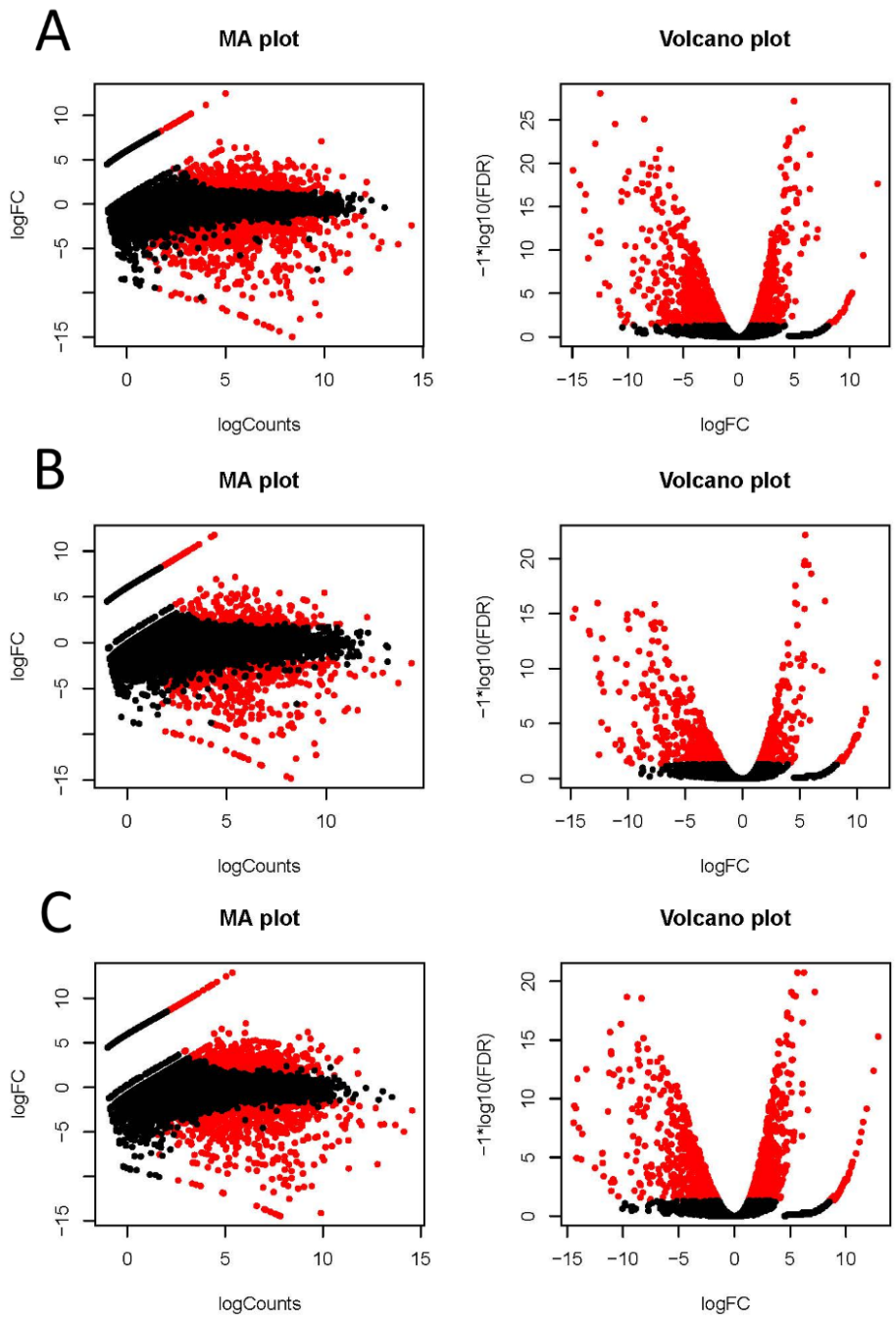


Figure 16: Volcano and MA plots for the *T. virens* DPI datasets.A) 3 DPI, **B**) 5 DPI and **C**) 7 DPI datasets. MA plots show logCounts vs LogFC (Log fold change). LogFC indicates the log of the change in expression compared to the control T. virens. Log counts are the log of the read counts mapping to each gene. The most robust data is indicated by points with the highest logCounts, whereas the most differentially expressed is indicated by those values at the extreme top or bottom of the map (indicating down or up regulation respectively). Red dots indicate transcripts that have statistically significant differential expression. Volcano plots show the LogFC vs the –log10FDR (False discovery rate), points with more fold change and higher –log10FDR are more reliable.

5.4.2 *T. virens* Three Days Post Inoculation

5.4.2.1 Proteins of Unknown Function

Proteins of unknown function make up the largest group and represent 10% of all proteins present in the *T. virens* proteome (**Figure 18**). Alignment of these proteins using ClustalW implemented in Mega 6 (Tamura et al., 2013) showed that these proteins are highly varied, with only 1 motif being identifiable in 2 separate proteins. MEME searches were performed on the unknown proteins to identify any smaller motifs that were not detectable in the sequence alignments. Three motifs were detected by MEME, the first of which (A) is a hydrophobin-like motif (**Figure 17**) (Wösten, 2001). Hydrophobins are implicated in fungal growth, particularly where the fungi must attach to a hydrophobic surface, such as a plant cuticle or cell wall, although it is notable that this motif contains 7 rather than the usual 8 cysteine residues common to major hydrophobins (Dubey, Jensen, & Karlsson, 2014; Seidl-Seiboth et al., 2011). It is possible however, that this is the result of an annotation error in the genome. Attachment to the plant surface is likely to be important in allowing *T. virens* to maintain itself on, and enter into plant root cell surfaces and it is likely that novel hydrophobins would play some role in this. Alternatively, cysteine rich proteins are likely to have strong internal bonding and stability against plant protease activity, which may enhance their survival in apoplastic fluid, or during effector delivery (Stergiopoulos & de Wit, 2009).

Figure 17: Motifs detected by MEME searches and CLUSTALW sequence alignments. Three separate motifs were detected by MEME searches of the up-regulated unknown protein DPI dataset. Motif A was detected in TV_79197 and 214571. Motifs B and C were both detected in TV_153126 and TV_210232. Motifs were located in similar positions near the end of the peptide in all of these proteins. Character sizes indicate their level of conservation in the motif. Dual letter combinations indicate either/or conservation at that site

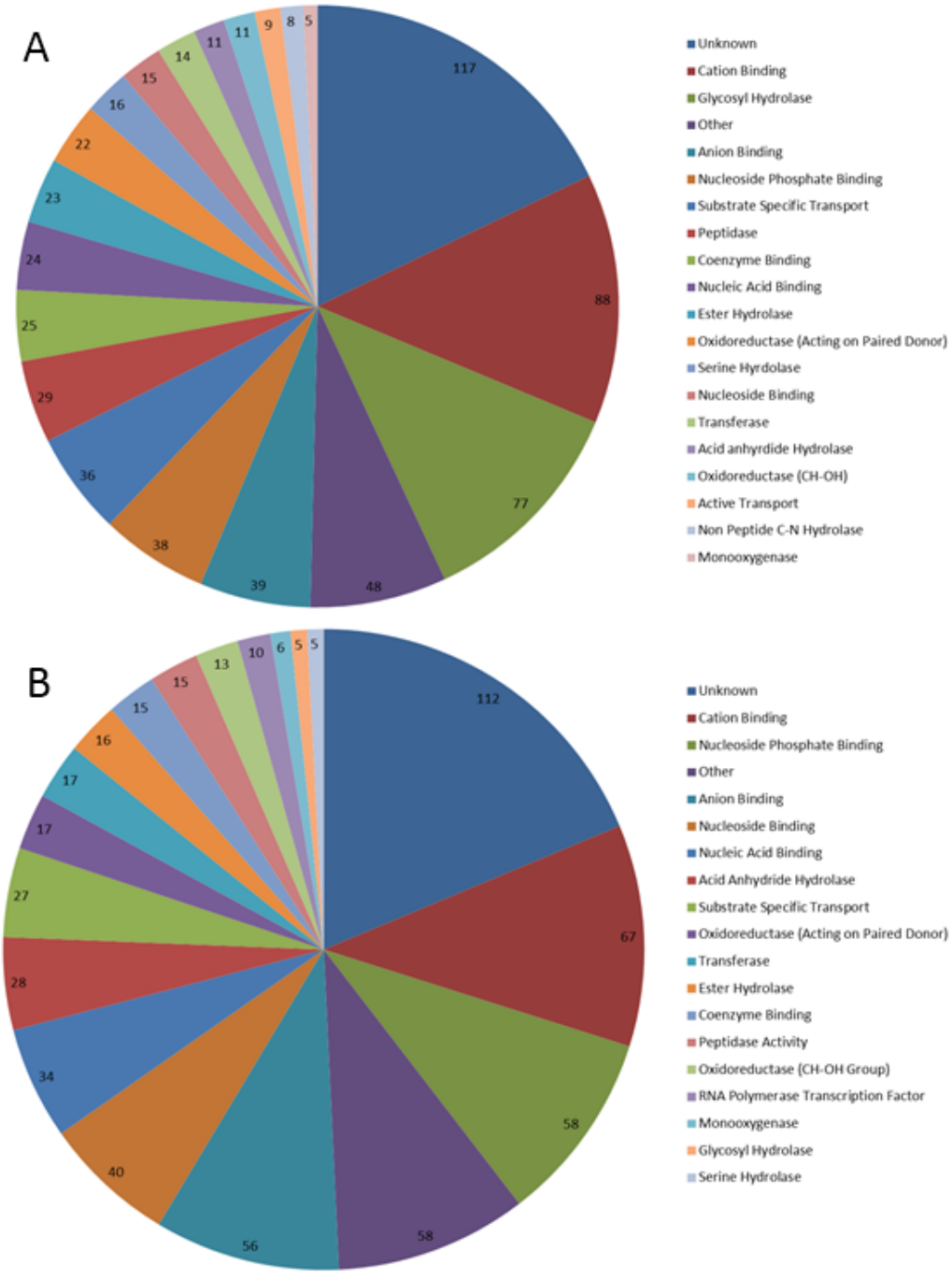


Figure 18: Functional categories of differentially expressed genes at 3 DPI. BLAST, Interpro and GO searches implemented in BLAST2GO were used to construct functional categories for up-regulated (A) and down-regulated (B) genes in the 3 DPI dataset. The number of genes present in each functional group is indicated inside the pie slices. Major functional groupings in both datasets included unknown and cation binding proteins. The major outlier was glycoside hydrolases with 77 up-regulated genes and only 5 down-regulated genes.

The remaining two motifs appear to be novel (**Figure 17,** B and C), giving little clue as to their function. It is important to note though, that owing to the large number (112) of unknown proteins

up-regulated in this interaction, it is likely that some of these encode effector proteins. A major challenge in understanding the molecular interactions between plants and root colonising fungi has been the relative abundance of novel genes with no detectable known motifs (Sperschneider et al., 2015). The few motifs known at this point include the oomycetes RxLR translocation and entry motif and the WxC motif of powdery mildew fungi, however nothing similar could be identified in our dataset (Godfrey et al., 2010; Tyler et al., 2013). The inability to predict the function of these molecules however, does not discount them as potential effectors. The effector-gene interaction follows a gene-for-gene model, or protein-complex vs receptor complex model (Gassmann & Bhattacharjee, 2012). It is likely that novel proteins form components of these receptor-ligand interaction complexes (Gassmann & Bhattacharjee, 2012; Jones & Dangl, 2006; Stergiopoulos & de Wit, 2009). This indicates that several of the unknown proteins may be involved in direct *T. virens*-maize interactions, and might be responsible for the specific phenotype on each maize line. The chief issue in this case is the inability to make rational predictions of effectors from 112 unknown proteins –it is likely that many of these proteins also fulfil unknown but biologically relevant functions. The proportion of unknown proteins up-regulated is approximately 10% (112 of 1021), which is similar to their total proportion in the genome. This suggests that perhaps only a select group of effectors are required for a particular host, or that few proteins can have a disproportionate effect on colonisation. Alternatively other pathways that are consistently up-regulated may be more important in root colonisation.

5.4.2.2 Cation Binding Proteins

Cation-binding proteins formed the second largest functional group, with 8.5% of these proteins being up-regulated. Cation binding functionalities were analysed to determine if a particular subset was overrepresented (**Figure 19**). Zinc ion binding appeared to be a major component of the up-regulated proteins. Zinc based proteins are often transcriptional regulators or recognition receptors and this suggests major transcriptional modification is occurring during root colonisation (Gupta et al., 2012; MacPherson et al., 2006). Zinc proteins have been shown to regulate processes such as cell adhesion in bacterial pathogens and to control hydrolase activity in other *Trichoderma* species (Aro, Saloheimo, Ilmén, & Penttilä, 2001; Panina, Mironov, & Gelfand, 2003). Particularly notable is the known regulation of several *T. reesei* cell wall degrading enzymes by the Cre1 repressor system (Ilmén, Thrane, & Penttilä, 1996; Takashima, Iikura, Nakamura, Masaki, & Uozumi, 1996). Cre1 encodes a zinc-finger protein responsible for the repression of cellobiohydrolase production; which

is important in light of the large scale up-regulation of these enzymes, discussed below. The increased regulatory activity is supported by the presence of DNA binding activity and DNA polymerase II transcription factors, which regulate transcriptional activity and responses to other signalling factors (Kadonaga, 2004). Increased regulation is predictable as the switch from a more generalist free-living lifestyle to a specialist endophytic one should require tight regulation of fungal processes and dramatic changes in feeding and growth behaviours.

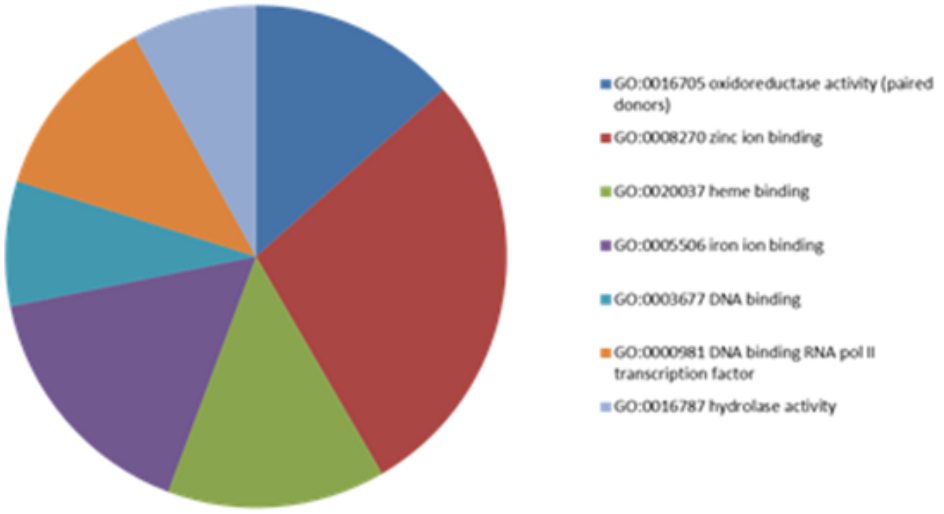


Figure 19: Distribution of cation binding protein types that were up-regulated at 3 DPI. Zinc and Heme binding proteins (red and green) made up just fewer than 50% of the cation binding proteins up-regulated at 3 DPI. Zinc-finger proteins are implicated in transcriptional regulation, whereas iron binding proteins may be responsible for scavenging iron from the host plant.

Heme ion binding is also a significant nutrient acquisition function in almost all living organisms (Harel, Bromberg, Falkowski, & Bhattacharya, 2014). Given the likely iron-deficient environment of a host plant, heme and iron ion binding proteins may have influence the ability of microorganisms to obtain nutrition in the host plant (Hao et al., 2012). It has been demonstrated in *Epichloë festucae* that disruption of iron-scavenging activity results in malformation of fungal hyphae and abnormal fungal localisation in the host plant (Johnson et al., 2013). Iron binding activity has also been detected in bacterial pathogens and endophytes, predominantly through the use of iron binding secondary metabolites such as siderophores (Hao et al., 2012; Lacava et al., 2008). The presence of numerous heme and iron binding functions provides indirect evidence that iron binding may be relevant in colonisation of the plant root. *T. virens* is known to express three types of siderophore (cis-fusarinine, trans-fusarinine and dimerum acid) that would facilitate this iron acquisition process (Jalal, Love, & van der Helm, 1986).

General effects of the up-regulation of 88 cation and 39 anion binding proteins may be in ion transport, osmotic balance and redox chemistry functions, which may be significant to root colonisation. Control of osmotic balance and redox chemistry is likely important in the ability of the fungus to defend itself from common plant defensive mechanisms such as oxidative bursts (Jones & Dangl, 2006). Tolerance to reactive oxidation species (ROS) generated in this matter has been demonstrated in *T. harzianum.* It was shown that during interactions with sunflower plants, the fungus could not only survive ROS damage, but could also enhance the host plant's resistance to oxidative damage (Brahma N. Singh, 2011). This indicates yet another close association between plant defence responses and endophytic activity.

Finally, concentration of metal ions to plant roots may have important effects on plant root nutrition and growth promotion, and *Trichoderma* species are already being used for their metal-ion concentrating abilities (Babu, Shim, Bang, Shea, & Oh, 2014). Metal-ion handling capability also allows the fungus to increase plant tolerance to adverse soil conditions (Babu, Shea, & Oh, 2014). Whilst probably not directly related to root colonisation ability, the up-regulation of this gene set may also explain some of the increased tolerance to abiotic effects demonstrated by plants inoculated with *Trichoderma* spp.

5.4.2.3 Lytic Enzymes and Carbohydrate Binding

Glycoside hydrolases form the next major up-regulated group and are overrepresented with 38% of the entire complement of these proteins up-regulated during the interaction. Hydrolases are the most highly up-regulated group, comprising approximately 60% of the top 50 most significantly up-regulated proteins (**Table 20**). Of these, the majority had glycosyl-bond hydrolysis or carbohydrate binding as their primary function (**Figure 20)**.

Glycoside hydrolases are likely to have significant roles in fungal nutrition within the plant host. Plant cell walls are composed of cellulose (glucose), hemicellulose (xylan, mannan, xyloglucan, glucomannan) and pectin (various galacturonans), which are all polymers comprised of various polysaccharides layered in such a way that they create a strong and digestion-resistant complex (Gilbert, 2010; Mohnen, 2008; Scheller & Ulvskov, 2010). The nutritional value of such a large polysaccharide and carbon source to a fungal endophyte seems obvious, yet it is also a significant obstacle, both in terms of its resistant to degradation and as a barrier to root penetration by the fungus. During the interaction *T. virens* up-regulated genes responsible for the degradation of

virtually every component of plant cell walls. Cellulose is targeted by proteins expressed from genes such as TV_90504 and TV_78675 (cellobiohydrolases), hemicellulases by xylan esterases such as TV_51211, TV_47222 and TV_39549 and pectin by enzymes such as TV_51095 (polygalacturonase, glycoside hydrolase family 28). Protective side chain polysaccharide groups can also be removed from the primary components by enzymes such as TV_48966 and TV_152027 (arabinofuranosidases, glycoside hydrolase family 62) (Abbott & Boraston, 2007; Degrassi, Okeke, Bruschi, & Venturi, 1998; E, A, R, G, & G, 1991; Gregg, 2011). Clearly *T. virens* has the potential for almost total breakdown of plant cell wall products, which is relatively unsurprising given its relative *T. reesei* is virtually the model organism for industrial cellulolytic biology. It is possible *T. virens* is even more capable given its expanded cell wall degradation potential.

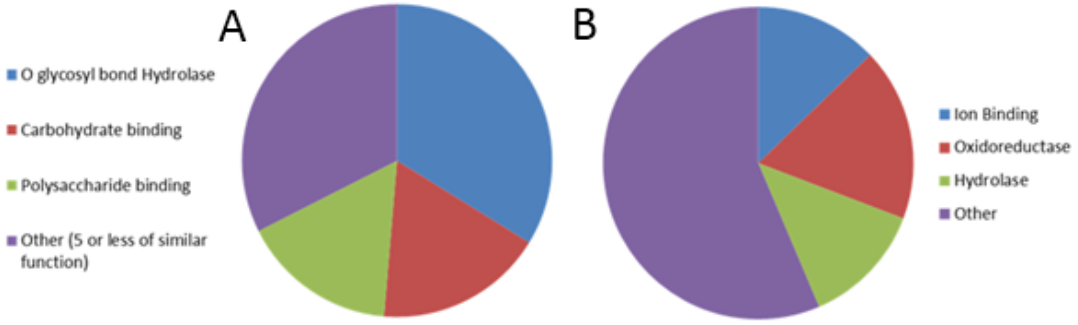


Figure 20: Functions of the top 50 most highly Up-regulated genes at 3 DPIA) The functions of the 50 up-regulated genes. B) The functional groupings of the 50 most down-regulated genes. Glycosyl bond hydrolases and polysaccharide degrading enzymes form a major portion of the most highly up-regulated proteins at 3 DPI. Several unknown proteins are also present, 4 of which are small enough to potentially be considered as effector proteins.

The prolific expression of lytic enzymes during root colonisation has three major functions. Firstly lytic enzymes allow breakdown of plant cell wall polymers into component polysaccharides, which may then be bound and metabolised (Z. Zhao, Liu, Wang, & Xu, 2013). The second function is weakening the plant cell wall, which allows fungal penetration, either by physical methods or by total degradation (Deising et al., 2000; Kubicek et al., 2014). The third is in plant-fungus communication by potential mechanisms such as cell-wall sensing, polysaccharide molecule based communication or damage associated molecular pattern (DAMP) pathways (Denness et al., 2011; Vargas, Mandawe, & Kenerley, 2009). The major caveat to this mechanism for the fungus, is the likelihood of triggering detrimental DAMP pathways (Beliën, Van Campenhout, Robben, & Volckaert, 2006). These lead to increased host resistance through methods such as cell wall lignification and callose deposition, triggered by the plant sensing damage or modification of its cell

wall (Bhuiyan, Selvaraj, Wei, & King, 2009; Caño-Delgado, Penfield, Smith, Catley, & Bevan, 2003; Hématy, Cherk, & Somerville, 2009). Cell wall degradation can explain the survival of an endophyte within its host and may also be partially responsible determining the extent of colonisation. Pathogenic fungi have been shown to have enzyme suites specifically suited to the degradation or outright destruction of the cell wall structure of particular hosts (e.g. cereal pathogens have more xylanases, necrotrophs have enhanced pectinase activity) (Annis & Goodwin, 1997; Hatsch, Phalip, Petkovski, & Jeltsch, 2006). It may be that endophytic CWDE's are not capable of dealing sufficient damage to the plant to have pathogenic effects; however this seems unlikely in the case of *T. virens*, as it appears to have all the functionalities necessary to completely degrade a plant cell. This would seem to suggest a regulatory process, probably linked to DAMP pathways, which limits the extent of *T. virens* damage to the plant cell wall, or prevents the plant from sensing the damage, although the fungus is still clearly deriving sufficient nutrition in the process.

Carbohydrate or polysaccharide binding functions of this group may also play a role in screening the fungus from detection by the plant. Scavenging or degradation of fungal breakdown products derived from plant proteases and lytic enzymes may prevent escalation of the plant immune response. LysM effectors have been demonstrated to scavenge chitin breakdown products, and given the large group of carbohydrate binding genes, it is possible that this functionality is extended in *T. virens* (de Jonge & Thomma, 2009). LysM appears to operate by chitin sequestration, but it is possible that lytic enzymes degrade fungal breakdown products in such a way that prevents their detection by the plant or that scavenging or active transport of breakdown products prevents their detection. It has also been demonstrated that LysM type proteins may be co-expressed with chitinases, suggesting that some carbohydrate binding proteins may be related to polysaccharide scavenging (Seidl-Seiboth et al., 2013). Overall the data suggests an important role for lytic enzymes and carbohydrate signalling in the maize-*Trichoderma* interaction.

Table 20: The 50 Most Highly Up-regulated genes at 3 DPI in *T. virens*

Seq. Identifier	Seq. Description	Seq Length*	LogFC**
TV_86039	**3-beta-hydroxysteroid dehydrogenase**	342	-8.65246
TV_72838	**Xylanase**	221	-11.1721
TV_51211	acetyl xylan esterase	299	-13.8382
TV_47222	acetyl xylan esterase	236	-10.9098
TV_39549	acetyl xylan esterase	230	-9.03411
TV_61793	aflatoxin biosynthesis ketoreductase nor1	248	-10.1495
TV_64881	aggrecan core protein	152	-9.51287
TV_62436	Alpha-galactosidase	409	-10.3055
TV_151988	Alpha-galactosidase 5 precursor	510	-12.7557
TV_154698	ankyrin repeat domain protein	313	-10.035
TV_32357	carbohydrate esterase family 15 protein	460	-12.507
TV_31131	carbohydrate esterase family 8 protein	329	-13.6026
TV_90504	**cellobiohydrolase I**	506	-12.528
TV_78675	**cellobiohydrolase II**	472	-8.5599
TV_53201	cytochrome p450	523	-12.601
TV_56652	**Endo-betaxylanase**	324	-14.976
TV_53264	Endo-betaxylanase	348	-10.6191
TV_182161	endoglucanase I	458	-8.75099
TV_222554	flagellar attachment protein	205	-9.21883
TV_8282	glycoside hydrolase family 11 protein	231	-13.314
TV_65505	glycoside hydrolase family 11 protein	227	-12.0644
TV_59409	glycoside hydrolase family 11 protein	231	-9.55357
TV_42536	glycoside hydrolase family 12 protein	236	-8.7697
TV_51095	**glycoside hydrolase family 28 protein**	381	-12.9633
TV_34797	glycoside hydrolase family 31 protein	767	-8.70747
TV_59335	glycoside hydrolase family 45 protein	243	-8.73992
TV_61403	glycoside hydrolase family 5 protein	444	-14.3467
TV_35701	glycoside hydrolase family 5 protein	424	-12.5731
TV_76400	glycoside hydrolase family 5 protein	420	-8.25455
TV_76869	glycoside hydrolase family 61 protein	348	-8.70124
TV_152027	glycoside hydrolase family 62 protein	379	-13.965
TV_48966	**glycoside hydrolase family 62 protein**	320	-9.23536
TV_59360	glycoside hydrolase family 74 protein	841	-8.1927
TV_32074	glycoside hydrolase family 92	442	-8.16898
TV_111696	**glycosyl hydrolase family 61**	252	-9.96401
TV_15704	gpi anchored protein	387	-9.58252
TV_150738	h k atpase alpha	1132	-11.7435
TV_43201	hypothetical protein	134	-10.6557
TV_49069	hypothetical protein	268	-9.54906
TV_52140	**hypothetical protein**	212	-10.5894

TV_66299	**hypothetical protein**	140	-9.35733
TV_34676	mfs sugar transporter	534	-10.0664
TV_34076	NRPS-like enzyme	1054	-8.53336
TV_63956	peptidase m35 deuterolysin	348	-8.15603
TV_47121	protein kinase	632	-8.03573
TV_123209	serine endopeptidase	910	-10.7428
TV_57302	sterol 24 c methyltransferase	386	-8.27984
TV_53145	terpene synthase	253	-9.04643
TV_192759	unknown protein	861	-10.2401
TV_70655	unknown protein	345	-8.14161
TV_52718	Zinc type alcohol dehydrogenase	348	-10.2524

Table 20: The 50 most highly up-regulated genes in *T. virens* at 3 DPI Gene descriptions as identified by BLAST searches are provided. Bold entries are also present in either the 5 or 7 DPI datasets. *Seq Length displays the length of the sequences in amino acids, which can be compared to the estimated size of effector proteins (fewer than 300 amino acids). **LogFC is the log fold change as compared to the control *T. virens* grown on minimal media. Note LogFC is shown as a negative value as it reads 'How down-regulated is the control compared to the sample. i.e. for TV_72838 the control has -11.17 expression compared to the sample.

5.4.2.4 Polysaccharide Transport Proteins

Supporting evidence for the role of the polysaccharide degrading molecules was the up-regulation of 16 polysaccharide transport molecules (**Table 21**). Maltose transport and multifunctional polysaccharide transport were the primary groups of sugar transporters identified. Two hexose transporter proteins were identified. Hexoses have been shown to interact with plant hormones to regulate signalling pathways and may also directly act as a signalling molecule (Eveland & Jackson, 2011; Moghaddam & Ende, 2012). Hexoses are thought to particularly regulate cell growth, proliferation and internal development (Eveland & Jackson, 2011). The ratio of sucrose to hexose in the plant cell has been shown to influence cellular responses; however no sucrose-specific transporters were up-regulated in this dataset (Tauzin & Giardina, 2014). It is also interesting to note that a related set of polysaccharide signalling proteins, the cell wall invertases, were also not differentially expressed at this time point. Also up-regulated are γ-aminobutyric acid (GABA) permeases. GABA accumulation was linked to cell death in rice cells and is inhibited during pathogenic infection with *M. oryzae* (Takahashi, Matsumura, Kawai-Yamada, & Uchimiya, 2008). This could indicate some repression of cell death by *T. virens* during colonisation.

Table 21: Up-regulated genes coding for sugar transporters at 3 DPI

Seq. Identifier	Seq. Description	Seq. Length*	LogFC**
TV_216420	**hexose transporter-like protein**	537	-2.14762
TV_78356	**hexose transport-related protein**	540	-2.42296
TV_28190	alpha-glucoside permease	554	-3.69282
TV_76958	**glucose transporter**	544	-2.61718
TV_66182	**glycerol uptake**	346	-3.28061
TV_57275	**high-affinity glucose transporter**	567	-7.16805
TV_78848	**lactose permease**	544	-4.63351
TV_38147	**maltose permease**	544	-5.39965
TV_32348	**maltose permease**	507	-4.26232
TV_28667	**maltose permease**	528	-2.9259
TV_68782	**maltose permease mal31**	534	-6.63531
TV_69846	**maltose permease mal31**	534	-2.50446
TV_34676	**mfs sugar transporter**	534	-10.0664
TV_27972	**mfs sugar transporter**	536	-3.81598
TV_53157	sugar transporters	537	-6.63557
TV_51260	sugar transporters	515	-3.23143

Maltose, hexose and multi-sugar transporters make up the predominant groups in this dataset. Note most transporters are over 300 amino acids in length. Bold entries are also up-regulated in either the 5 or 7 DPI dataset. *Seq Length displays the length of the sequences in amino acids, which can be compared to the estimated size of effector proteins (fewer than 300 amino acids). **LogFC is the log fold change as compared to the control T. virens grown on minimal media. Note LogFC is shown as a negative value as it reads 'How down-regulated is the control compared to the sample

5.4.2.5 Secondary Metabolites

Secondary metabolites include hormones, toxins and bioactive compounds, a number of which were up-regulated at 3 DPI (**Table 22**). These can have systemic effects on the regulation of plant pathways, including those responsible for growth and innate immunity. 1-aminocyclopropane-1-carboyxlate synthases (ACC synthases) are responsible for the production of ethylene (K. L.-C. Wang, Li, & Ecker, 2002). Ethylene is a major regulator of plant growth and immunity that regulates the plant response to biotic and abiotic stress. Two of these proteins, TV_44837 and TV_127285 were up-regulated at this time point. Expression of these in *T. virens* may indicate the fungus is interfering with plant ethylene regulation, and this has been observed in the interaction of *Trichoderma viride* with *Nicotiana tabacum* (Avni, Bailey, Mattoo, & Anderson, 1994).

Another important group, that also supports the important of cell wall interactions, is the quinates. These are highly bioactive molecules that are also precursors to a number of biosynthetic pathways. They are often associated with lignin production in plants, in response to a microbial invader (Bhuiyan et al., 2009). As the majority of up-regulated quinate products are quinate permeases, it is

possible that *T. virens* may be degrading plant cell wall lignins and actively transporting and metabolizing their breakdown products (Igor Cesarino, 2012). Lignins may also be catabolised as an energy source via a specific degradation pathway, involving the enzymes quinate dehydrogenase, 3-dehydroquinase and dehydroshikimate dehydratase (Wheeler, Lamb, & Hawkins, 1996). This pathway also requires redox chemistry, particularly that of heme carrying molecules, to function, which may be provided by the extensive up-regulation of metal ion genes discussed previously (Dashtban, Schraft, Syed, & Qin, 2010). TV_57148 (3-dehydroquinase), TV_42357 (quinate dehydrogenase) and TV_42027 (dehydroshikimate dehydratase) are all up-regulated at this time, strongly suggesting quinate catabolism as a nutrition method for *T. virens*. This also allows the fungus to derive nutrition from cell-wall lignification, and may aid it in penetrating the plant host.

Several other secondary metabolite biosynthetic proteins appear to be up-regulated in this dataset. Aflatoxins are highly toxic proteins generally associated with *Aspergillus* fungi, and although they have no currently known role in immune modulation, they are strongly cytotoxic and may also act as elicitors in some host plants (Klich, 2007; Z. Wang, Yan, Liu, Chen, & Wang, 2012). Kynurenines are known to regulate a large number of immune pathways in animals and have been directly linked to hormonal regulation in *Arabidopsis*, where they interact with ethylene and auxin signalling pathways (He et al., 2011; Wilson, Thomsen, Petersen, Duus, & Oliver, 2003).

Sterols, terpenes and polyketides made up the remainder of the secondary metabolites up-regulated at 3 DPI. These are very large families of compounds with a wide variety of biological activity. Almost all are bioactive and pathway products from each family have been directly linked to plant immunity. In oranges, down-regulation of terpenes has been shown to promote resistance to fungal pathogens (Rodríguez et al., 2014). Sterols produced by Trichoderma spp. have been shown to have antibiotic activity, particularly against other fungi, and are also important precursors of many hormones (Gräfe et al., 1991). Sterols also have functions in endocytosis, via the constructions of lipid-based secretory bodies (Geldner, 2004). Sterols of the 24-C-methyltransferase type (TV_57302) are strongly linked to both hormone biosynthesis and fungal cell wall engineering, whilst also being a precursor to antioxidant compounds such as vitamin D (Weete, Abril, & Blackwell, 2010). All three of these functions may have roles in fungal root colonisation, whether by modulation of plant hormones, modification or construction of fungal cell walls, or prevention of oxidative damage.

Table 22: Up-regulated genes coding for hormones, secondary metabolites and toxins at 3 DPI

Seq. Identifier	Seq. Description	Seq. Length*	LogFC**
TV_80487	4-coumarate- ligase protein	552	-3.03068
TV_44837	acc synthase	439	-1.57969
TV_127285	acc synthase	442	-3.69069
TV_79352	aflatoxin b1 aldehyde reductase member 2	351	-1.95553
TV_61793	aflatoxin biosynthesis ketoreductase nor-1	248	-10.1495
TV_52152	aflatoxin biosynthesis ketoreductase nor-1	248	-5.7534
TV_44396	GABA permease	496	-3.41813
TV_56645	GABA permease	509	-2.5027
TV_90363	kynurenine 3-monooxygenase	505	-1.77532
TV_47671	kynurenine aminotransferase	430	-3.92733
TV_73281	kynurenine formamidase	295	-1.80101
TV_34796	MFS quinate	568	-2.10704
TV_47828	polyketide synthase	2506	-2.90069
TV_42357	quinate dehydrogenase	318	-3.34124
TV_66077	quinate permease	543	-4.5065
TV_32748	quinate permease	513	-3.30132
TV_53789	quinate permease protein	528	-7.43183
TV_57302	sterol 24-C-methyltransferase	386	-8.27984
TV_36785	sterol desaturase family protein	257	-2.27485
TV_53145	terpene synthase metal binding domain protein	253	-9.04643
TV_56195	terpene synthase metal binding domain protein	378	-5.60085

Secondary metabolite related proteins up-regulated in *T. virens* at 3 DPI. LogFC shows the log fold change in expression of the control compared to the sample. I.e. coumarate ligase is expressed 3x more in the sample than in the control. Items in bold are up-regulated in at least one of the 5 DPI or 7 DPI datasets. *Seq Length displays the length of the sequences in amino acids, which can be compared to the estimated size of effector proteins (fewer than 300 amino acids). **LogFC is the log fold change as compared to the control T. virens grown on minimal media. Note LogFC is shown as a negative value as it reads 'How down-regulated is the control compared to the sample

Polyketide production in *Trichoderma* spp. is extensive, and their vast arsenal of these chemicals may have significant roles in root colonisation (Degenkolb, von Döhren, Nielsen, Samuels, & Brückner, 2008). Polyketides have been implicated as avirulence factors in *Magnaporthe grisea,* suggesting a role in pathogenicity, however production of these by *T. virens* would appear counterproductive if that were the case in this interaction (Bohnert et al., 2004). Disruption of polyketide synthase activity has been shown to impair the ability of *T. virens* to induce systemic resistance in plants, suggesting that metabolites produced by this pathway have an important role in plant-fungus communication (Mukherjee, Buensanteai, et al., 2012). Activity of genes of this type has also been linked to the ability of mycorrhizal fungi to successfully establish a relationship with a plant host (Bonfante & Requena, 2011). Polyketide pathways are also involved in the production of iron scavenging siderophores, which have been implicated as important nutritional factors for

fungal colonisation (Johnson et al., 2013; Mukherjee, Horwitz, & Kenerley, 2012). Production of the trichocethene toxins is also controlled by polyketides pathways, and a secondary gene (TV_47880) related to production of these was up-regulated, although this gene is associated with fungal resistance to antibiotics (Kimura et al., 1998). It is clear that polyketide pathways have a huge range of functionalities relevant to root colonisation; however the major difficulty with these compounds is isolating their functions given the scale and complexity of their production in *Trichoderma*.

5.4.2.6 Transcription Factors

Transcription factors are major regulatory proteins in fungal species. In the 3 DPI dataset, the C6 transcription factors and fungal specific regulators formed the major up-regulated groups (**Table 23**). Three transcription factors with known functions were also identified in this dataset.

C6 transcription factors are one of the most diverse groups present in eukaryotic organisms. In *T. reesei* C6 transcription factors have been linked to regulation of lytic enzymes such as cellulases, hemicellulases and xylanases (Hakkinen et al., 2014). Regulation of these enzymes is also supported by the presence of Xlnr1 (TV_58714), which is a known C6 type transcriptional regulator of CWDEs (Peij, Gielkens, Vries, Visser, & Graaff). This protein specifically regulates xylanase production in *Aspergillus niger*. Pro-1 is another C6 type transcription factor. This is associated with sexual development in filamentous fungi and has been linked to fruiting body development in *Sordaria macrospora* (Sandra Masloff, Jacobsen, Pöggeler, & Kück, 2002; S. Masloff, Pöggeler, & Kück, 1999). Yrr1 is also of the C6 transcription factor family and is known to regulate drug resistance genes(Le Crom et al., 2002, p. 1). C6 transcription factors have been shown to have regulatory effects determined by oxidative stress. In *Aspergillus nidulans* and *C. cerevisiae* C6 transcription factors initiate production of redox proteins (such as superoxide dismutase), as well as initiating the production of protective secondary metabolites (Aguirre, Hansberg, & Navarro, 2006; Hong, Roze, & Linz, 2013). In particular non-ribosomal peptide synthases, which are commonly hybrid molecules related to the polyketides pathway, are often involved in this activity – and are highly up-regulated as discussed above (Lee et al., 2005).

C6 transcription factors have functions linked to many of the genes up-regulated in this dataset. In particular the presence of lytic enzyme regulatory genes provides supporting evidence for the role of these molecules during the plant-fungus interaction. The possible function of these transcription

factors in regulation of secondary metabolite pathways is also significant, and provides some regulatory framework for the transcriptional changes observed in this study.

Table 23: Up-regulated genes coding for transcription factors at 3 DPI

Seq. Identifier	Seq. Description	Seq. Length*	LogFC**
TV_124741	C6 transcription	630	-1.674798345
TV_113696	C6 transcription factor	631	-2.101864671
TV_134723	C6 transcription factor	507	-1.808157674
TV_44179	family transcriptional regulator	311	-3.397465082
TV_31797	family transcriptional regulator	311	-1.49163716
TV_124588	fungal specific transcription factor domain	618	-2.451351083
TV_135256	fungal specific transcription factor domain	458	-2.254397192
TV_229241	fungal transcriptional regulatory protein	851	-2.036361155
TV_58714	transcriptional activator xlnr1	922	-1.886145429
TV_51254	transcriptional protein	264	-3.502502375
TV_232488	transcriptional regulatory protein pro-1	632	-1.591600934
TV_42523	zinc finger transcription factor yrr1	710	-1.734224682

Up-regulated transcription factors at 3 DPI are shown here. *Seq Length displays the length of the sequences in amino acids, which can be compared to the estimated size of effector proteins (fewer than 300 amino acids). **LogFC is the log fold change as compared to the control T. virens grown on minimal media. Note LogFC is shown as a negative value as it reads 'How down-regulated is the control compared to the sample I.e. the control is 1.6x down-regulated compared to the sample for TV_124741. Entries in bold are also present in a least one of the 5 DPI or 7 DPI datasets.

5.4.2.1 Down-regulated *Trichoderma* Genes

Patterns in the down-regulated datasets were much harder to identify. The top down-regulated genes were comprised of a large number of functionalities that formed few clear groups. The reduction in oxidoreductases is intriguing in that it might be expected that these would help protect the fungus from oxidative bursts, but may be explainable as oxidoreductases linked to inactive metabolic processes may simply be switched off. It could also indicate that fungus is capable of preventing or mitigating plant oxidative burst responses, therefore having less need for activation of extra protective genes. Endophytic fungi and bacteria are known to be capable of mitigating the plant oxidative response in this manner (Bordiec et al., 2011; Hilbert, Nostadt, & Zuccaro, 2013). The down-regulated hydrolases are largely from the acid anhydride group rather than the glycoside hydrolases. This suggests that hydrolysis of other metabolites are less significant during the interaction, and indirectly supports the lytic enzyme pathway as a primary form of nutrition during endophytic growth.

Clustering analysis was performed to determine if groups with similar expression patterns had related functionalities. Clusters were readily observable in the up-regulated but not the down-

regulated data set (**Figure 21**). Examination of the most significantly up-regulated cluster showed some linkage of functionality (**Table 20**), with a number of CWDEs being present, alongside an mfs sugar transporter.

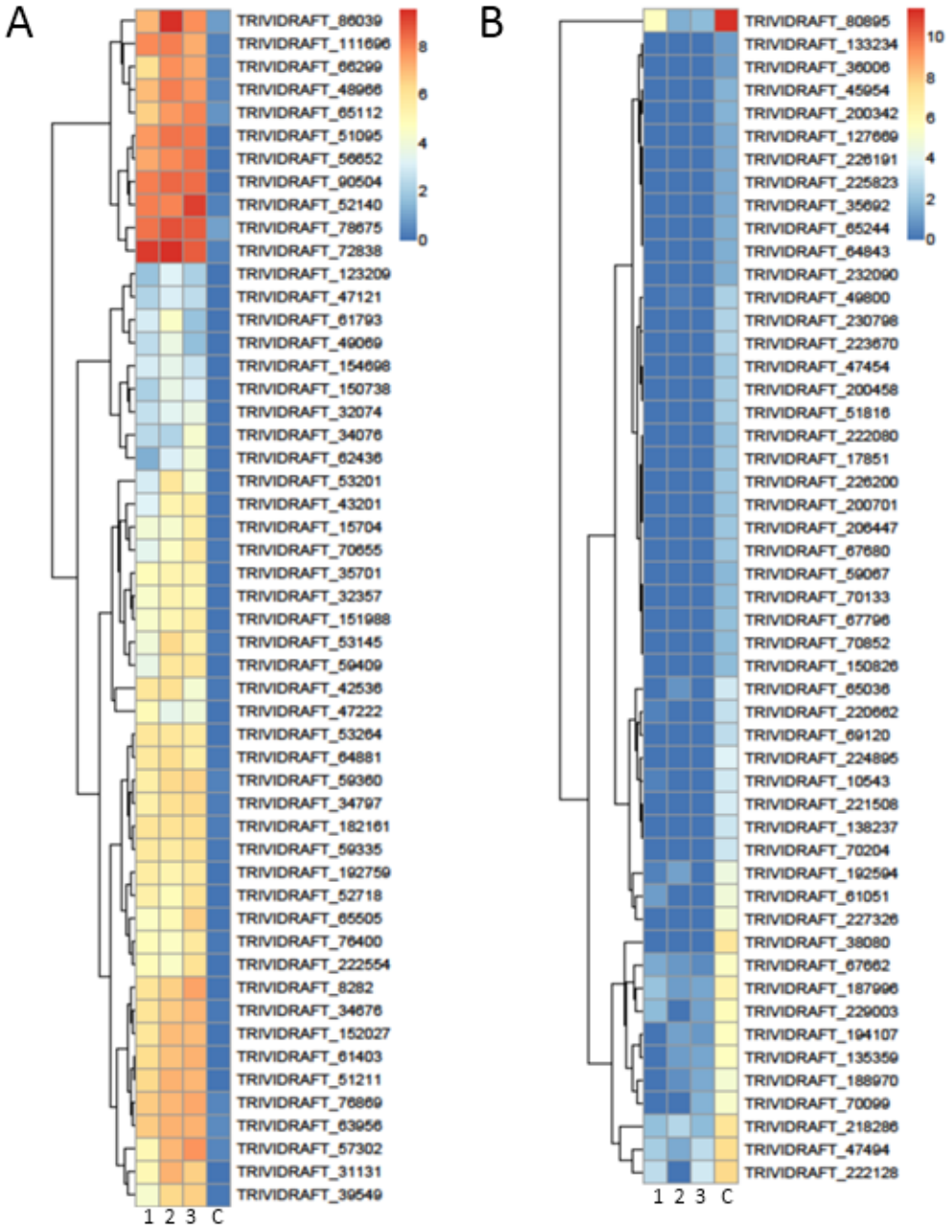


Figure 21: A clustered heat plot showing differential expression in *T. virens* at 3 DPI.Heatplots were created from FPKM values created from LogFC and count data. The top 50 most up-regulated (A) and top 50 most down-regulated (B) genes at 3 DPI are shown. Each column represents one sample (1, 2, and 3). C stands for control. The rightmost column shows the expression level in the control (T. virens on minimal media). Red represents the highest expression, blue represents the lowest. Note the top most cluster shows consistent strong up-regulation across all samples. This graphic can be compared with Table 17 for more specific detail. TRIVIDRAFT is the alternate JGI identifier code from TV_.

5.4.3 *T. virens* Five Days Post Inoculation

The 5 DPI time point followed similar trends to those observed at 3 DPI. Up-regulation of unknown proteins and glycoside hydrolases was again the predominant response (**Figure 22** and **Table 24**). Xylanases and cellulases were again amongst the most strongly up-regulated genes. Three acetyl xylan esterases were also up-regulated. These are responsible for deacetylation of xylan molecules which allows xylanases to degrade them (Sinha, Gupta, Patel, Ranjan, & Sonti, 2013). The sugar transporters detected in the previous sample no longer clustered with these genes at 5 DPI; however 4 sugar transporters were still amongst those most highly up-regulated in this data set.

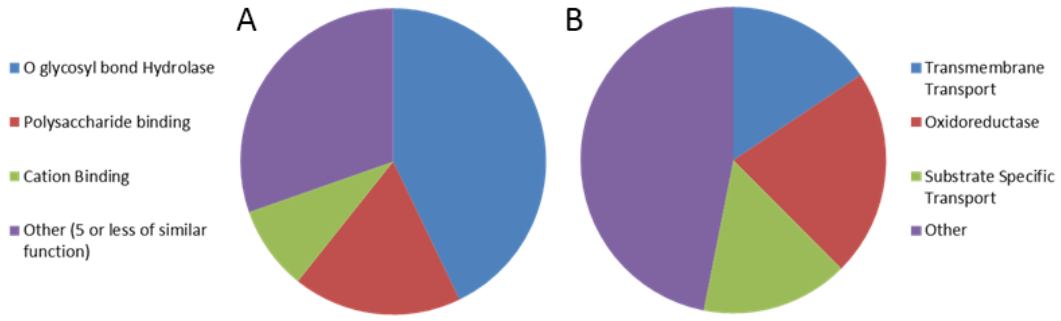


Figure 22: Functional breakdown of the Top 50 differentially expressed genes at 5 DPI.A) The functions of the 50 up-regulated genes. B) The functional groupings of the 50 most down-regulated genes. Glycosyl bond hydrolases and polysaccharide degrading enzymes clearly form a major segment of the most highly up-regulated proteins at 5 DPI as in the 3 DPI dataset.

Nine ankyrin repeat proteins were up-regulated at this time point. These proteins are commonly involved in protein-protein interactions and signalling and are known to act as transcriptional regulators of pathogenesis factors, such as toxins, in other fungi (Mosavi, Cammett, Desrosiers, & Peng, 2004). One of these was amongst the most highly up-regulated proteins in the data set.

Intriguingly two proteins related to the aflatoxin biosynthesis pathway (TV_61793 and TV_52152) were also up-regulated. Aflatoxins are linked to pathogenesis and are capable of provoking an immune response from the host plant (Z. Wang et al., 2012). Aflatoxins are also damaging to human health and may be of concern if *Trichoderma* is to be used as a bio control agent. The expression of aflatoxin during an endophytic relationships is surprising, and may reflect the opportunistic nature of *T. virens* endophytic ability – the endophytism between it and this host is not perfectly developed in evolutionary terms (Kubicek et al., 2011).

Table 24: The 50 most highly up-regulated genes at 5 DPI in *T. virens*

Seq. Identifier	Seq. Description	Seq Length*	LogFC**
TV_51211	acetyl xylan esterase	299	-13.4045
TV_47222	acetyl xylan esterase	236	-9.8928
TV_39549	acetyl xylan esterase	230	-8.8026
TV_61793	aflatoxin biosynthesis ketoreductase nor-1	248	-10.7163
TV_64881	aggrecan core protein	152	-9.4376
TV_62436	alpha-galactosidase	409	-10.4778
TV_151988	alpha-galactosidase 5 precursor	510	-12.4296
TV_154698	ankyrin repeat domain-containing protein 50	313	-12.3250
TV_223654	ankyrin repeat protein	473	-9.0433
TV_32357	carbohydrate esterase family 15 protein	460	-12.1350
TV_31131	carbohydrate esterase family 8 protein	329	-12.5217
TV_90504	**cellobiohydrolase I**	506	-12.2660
TV_78675	**cellobiohydrolase II**	472	-8.5223
TV_53201	cytochrome p450	523	-12.5433
TV_53264	endo-beta-xylanase	348	-10.6578
TV_56652	**endo-beta-xylanase**	324	-14.9760
TV_182161	endoglucanase i	458	-8.3184
TV_62437	fasciclin domain protein	214	-8.9670
TV_222554	**flagellar attachment zone protein 1**	205	-10.2119
TV_8282	glycoside hydrolase family 11 protein	231	-12.7737
TV_65505	glycoside hydrolase family 11 protein	227	-11.1172
TV_59409	glycoside hydrolase family 11 protein	231	-8.8206
TV_42536	glycoside hydrolase family 12 protein	236	-8.6749
TV_51095	**glycoside hydrolase family 28 protein**	381	-12.6608
TV_34797	glycoside hydrolase family 31 protein	767	-9.2784
TV_59335	glycoside hydrolase family 45 protein	243	-8.6665
TV_61403	glycoside hydrolase family 5 protein	444	-14.6085
TV_35701	glycoside hydrolase family 5 protein	424	-11.7650
TV_76869	glycoside hydrolase family 61 protein	348	-8.8089
TV_152027	glycoside hydrolase family 62 protein	379	-13.3258
TV_32074	glycoside hydrolase family 92	442	-9.0172
TV_111696	**glycosyl hydrolase family 61**	252	-9.4567
TV_15704	GPI anchored	387	-10.7780
TV_150738	h k ATPase alpha	1132	-12.3516
TV_68245	hydrophobin 3 precursor	109	-8.3918
TV_43201	hypothetical protein	134	-10.1080
TV_52140	**hypothetical protein**	212	-9.9133
TV_64880	hypothetical protein	213	-9.6857
TV_66215	hypothetical protein	254	-9.8681

TV_66299	hypothetical protein	140	-8.7591
TV_34676	mfs sugar transporter	534	-10.0639
TV_47121	protein kinase	632	-9.2582
TV_53145	terpene synthase metal binding protein	253	-8.9540
TV_192759	unknown protein	861	-10.0988
TV_59074	unknown protein	466	-9.9930
TV_202300	unknown protein	340	-9.0974
TV_205870	unknown protein	292	-8.6837
TV_205868	unknown protein	340	-8.3404
TV_72838	**Xylanase**	221	-11.0278
TV_52718	zinc-type alcohol dehydrogenase	348	-10.1407

Table 24 shows the 50 most highly up-regulated genes in *T. virens* at 5 DPI and their descriptions as identified by BLAST searches. *Seq Length displays the length of the sequences in amino acids, which can be compared to the estimated size of effector proteins (fewer than 300 amino acids). **LogFC is the log fold change as compared to the control *T. virens* grown on minimal media. Note LogFC is shown as a negative value as it reads 'How down-regulated is the control compared to the sample. I.e. for TV_51211 the control has -13.4 expression compared to the sample.

A range of volatile biosynthesis pathways appear to become up-regulated at the 5 DPI time point. Terpene, sterol and flavin pathways all had genes up-regulated. Volatile compounds are capable of a wide range of functions, such as nutritional regulation, modulation of plant immunity and cellular toxicity (Rodríguez et al., 2014; K. Wang, Senthil-Kumar, Ryu, Kang, & Mysore, 2012). A number of both quinone and quinate related proteins were also up-regulated at this point. These molecules are important precursors to a number of biosynthetic pathways (such as the terpene pathway).

Several antibiotic resistance genes, predominantly for bleomycin resistance, were produced at this time point. According to the gramene database (http://pathway.gramene.org/MAIZE/NEW-IMAGE?object=Bleomycins) maize is capable of expressing bleomycins. Expression of resistance genes could indicate a direct response to antibiotic attack by *T. virens*. This may also indicate that maize is still capable of detecting *T. virens* as an invading microbe at 5 DPI.

Proteins of unknown function were once again a prevalent up-regulated group as 5 DPI. Fifty of the unknown proteins up-regulated in the 3 DPI sample remained up-regulated at this time point, suggesting that these may be more important to the interaction. The 50 proteins conserved between datasets were examined for motifs using MEME and CLUSTALW alignment, however only the motifs previously identified in the 3 DPI set were again detected. The lack of new motifs and unknown function of these proteins again demonstrates the difficulty of identifying effector functions from these groups.

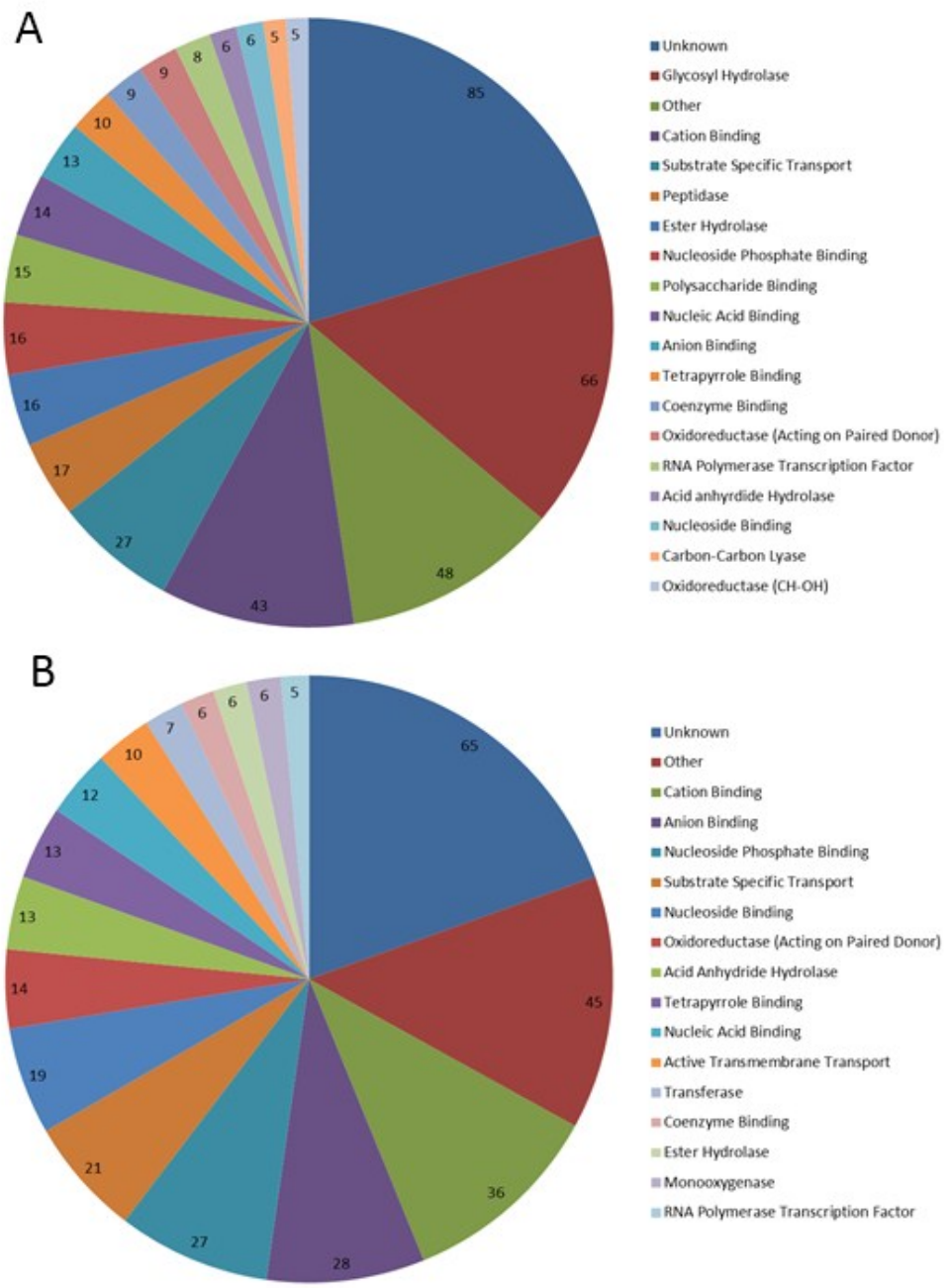


Figure 23: Functional categories of all differentially expressed genes at 5 DPI. BLAST, Interpro and GO searches implemented in BLAST2GO were used to construct functional categories for up-regulated (A) and down-regulated (B) genes in the 5 DPI dataset. The number of genes present in each functional group is indicated inside the pie slices. Unknown proteins and Glycosyl hydrolases were again the most significantly up-regulated groups.

5.4.4 *T. virens* Seven Days Post Inoculation

Trends in the 7 DPI time point differ slightly from that in the previous samples. Cation binding functionalities appear to be more important than at previous time points, however up-regulation of glycoside hydrolases is maintained (**Figure 25**). A large percentage of the most highly up-regulated genes were glycoside hydrolases.

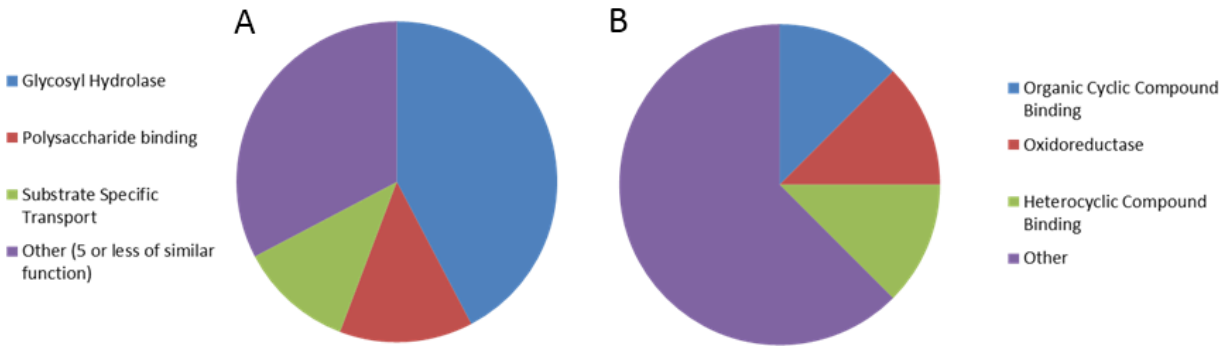


Figure 24: Functionalities of the top 50 differentially expressed genes at 7 DPI.. A) The functions of the 50 up-regulated genes. B) The functional groupings of the 50 most down-regulated genes. Glycosyl bond hydrolases and polysaccharide degrading enzymes remain the major up-regulated group.

Cluster analysis at this time point was less meaningful with only unrelated proteins having similar expression patterns (**Figure 24** and **Table 25**). The number of substrate transporters up-regulated at this time point showed a dramatic increase from the 5 DPI dataset (38 vs 27). This may suggest stabilisation of the plant-fungal relationship at this time point, or an increased amount of fungal tissue being involved in nutrition acquisition within the host plant.

Of the highly up-regulated protein cluster shown above, the gibberellin oxidase appears to be the most interesting protein. Gibberellins are linked to plant growth regulation and possibly defence signalling in plants via DELLA pathway mediated interactions (Davière & Achard, 2013; Lo et al., 2008; Mańka, 1980). Gibberellin expression has also been observed in fungal pathogens such as *Fusarium* (Troncoso et al., 2010). Several *Trichoderma* species are capable of acting as growth promoters and this may be linked to that functionality (Brotman et al., 2013; Hermosa, Viterbo, Chet, & Monte, 2012).

Table 25: The 50 most highly up-regulated genes at 7 DPI in *T. virens*

Seq. Identifier	Seq. Description	Seq Length*	LogFC**
TV_58493	acetyl esterase	346	-9.36504
TV_51211	acetyl xylan esterase	299	-14.4472
TV_47222	acetyl xylan esterase	236	-11.8595
TV_39549	acetyl xylan esterase	230	-8.85901
TV_64881	aggrecan core protein	152	-8.54497
TV_62436	alpha-galactosidase	409	-10.4425
TV_151988	alpha-galactosidase 5 precursor	510	-13.3154
TV_32357	carbohydrate esterase family 15 protein	460	-11.891
TV_31131	carbohydrate esterase family 8 protein	329	-13.9711
TV_90504	cellobiohydrolase I	506	-11.0739
TV_56652	endo-beta-xylanase	324	-14.976
TV_62437	fasciclin domain protein	214	-8.98521
TV_222554	flagellar attachment zone protein 1	205	-9.68276
TV_93146	**gibberellin 2-oxidase**	358	-9.10586
TV_58257	glucan endo-beta-glucosidase b	409	-8.7677
TV_8282	glycoside hydrolase family 11 protein	231	-14.1858
TV_65505	glycoside hydrolase family 11 protein	227	-13.7568
TV_59409	glycoside hydrolase family 11 protein	231	-11.7612
TV_51095	**glycoside hydrolase family 28 protein**	381	-14.1116
TV_34797	glycoside hydrolase family 31 protein	767	-9.39343
TV_61403	glycoside hydrolase family 5 protein	444	-13.6822
TV_35701	glycoside hydrolase family 5 protein	424	-10.9116
TV_152027	glycoside hydrolase family 62 protein	379	-14.2506
TV_48966	glycoside hydrolase family 62 protein	320	-9.25104
TV_48997	glycoside hydrolase family 79 protein	518	-10.299
TV_32074	glycoside hydrolase family 92	442	-9.31428
TV_49694	glycosyl hydrolase	799	-9.18318
TV_111696	glycosyl hydrolase family 61	252	-8.53587
TV_15704	gpi anchored	387	-11.2499
TV_150738	H+K+ ATPase alpha	1132	-11.8412
TV_57275	high-affinity glucose transporter	567	-8.68866
TV_68245	**hydrophobin 3 precursor**	109	-11.3681
TV_45154	**hypothetical protein**	126	-10.1919
TV_52140	hypothetical protein	212	-10.1208
TV_49120	hypothetical protein	506	-8.66785
TV_217788	hypothetical protein	86	-8.64982

TV_79197	hypothetical protein	124	-8.63836
TV_128086	hypothetical protein	258	-8.60943
TV_222460	hypothetical protein	364	-10.8109
TV_41854	**hypothetical protein**	170	-9.67513
TV_34676	mfs sugar transporter	534	-10.3841
TV_14392	partial	434	-8.69791
TV_53789	quinate permease protein	528	-8.67874
TV_34877	related to hard surface induced protein 3	520	-11.0846
TV_194392	Unknown Protein	972	-11.1738
TV_59074	Unknown Protein	466	-11.0738
TV_192759	Unknown Protein	861	-10.8733
TV_51972	williams-beuren syndrome protein	213	-8.51016
TV_72838	**Xylanase**	221	-11.0962
TV_52718	zinc-type alcohol dehydrogenase	348	-12.531

The 50 most highly up-regulated genes in *T. virens* at 7 DPI and their descriptions as identified by BLAST searches. *Seq Length displays the length of the sequences in amino acids, which can be compared to the estimated size of effector proteins (fewer than 300 amino acids). **LogFC is the log fold change as compared to the control *T. virens* grown on minimal media. Note LogFC is shown as a negative value as it reads 'How down-regulated is the control compared to the sample. i.e. for TV_58493 the control has -9.36 expression compared to the sample.

Several responses consistent with the 3 and 5 DPI samples were observed. Glycoside hydrolases and acetyl xylan esterases were up-regulated, continuing the theme of polysaccharide degradation as a primary response. Polysaccharide transport appeared more diversified at this time point, with hexose, mannose and maltose related proteins becoming more prevalent. Ankyrin repeat proteins were again present in relatively large numbers alongside several other fungal transcription factors. One aflatoxin biosynthesis pathway enzyme was up-regulated at this time point.

Volatile compounds produced differed slightly from earlier samples, with coumarate, shikimate and kynurenine production being up-regulated. These are derivatives of phenylalanine, tryptophan and tyrosine biosynthesis (Herrmann, 1995). The pathways are associated with the production of a large number of metabolically active derivatives in both plants and fungi, including lignin, flavonoids and alkaloids (Herrmann, 1995; Tzin & Galili, 2010). Approximately 12,000 plant alkaloids are known, most of which are highly bioactive, commonly being involved in antibiosis and plant defence (Facchini, 2001). Endophytic fungi may be able to use these pathways to imitate plant signalling pathways and active compounds, thereby controlling plant responses to suit their needs (N. Sachin, 2013). Alkaloids are already known to be produced by endophytic fungi, in what appears to be a defensive arrangement with their host plant, although the extent of the endophyte's contribution appears uncertain (Rasmussen, Parsons, Popay, Xue, & Newman, 2008; Schardl, Grossman,

Nagabhyru, Faulkner, & Mallik, 2007). It has also been established that alkaloids contribute to the benefits endophytes confer to a host's abiotic stress tolerance (Bush, Wilkinson, & Schardl, 1997).

Coumarate ligases are responsible for a production of a wide range of diverse secondary metabolites and are up-regulated in plants upon response to fungal elicitors, possibly suggesting pathway derivatives are involved in cross-communication (Kuhn, Chappell, Boudet, & Hahlbrock, 1984). The shikimate pathway is responsible for an incredibly diverse array of volatiles, of which many are involved in plant defence (Tzin & Galili, 2010). It is also linked to the coumarate pathway and these may thus be supporting each other. Kynurenines have been shown to be produced by the pathogenic fungi *Blumeria graminis* and may be responsible for adhesion to the plant (Wilson et al., 2003). Given the large scale of simple carbon polysaccharide scavenging from the plant, it may also be possible that these biosynthetic pathways are activated as the fungus begins to produce such products from plant polysaccharides, rather than obtaining them from the environment.

Down-regulated genes were again less consistent in their functional groupings. However several interesting proteins were down-regulated. TV_48225 and TV_48610 which encode for an aflatoxin biosynthesis enzyme and an aflatoxin efflux pump were both down-regulated at this time point. The down-regulation of the efflux pump is intriguing given the consistent up regulation of the synthesis enzymes in all previous datasets. Two gliotoxin biosynthesis proteins and the previously identified HC toxin biosynthesis enzyme were also down-regulated, hinting at a reduced role for toxic compounds at longer interaction points.

A large number of ankyrins, transcription factors and signalling proteins were down-regulated, potentially indicating a change in regulatory patterns at this time point.

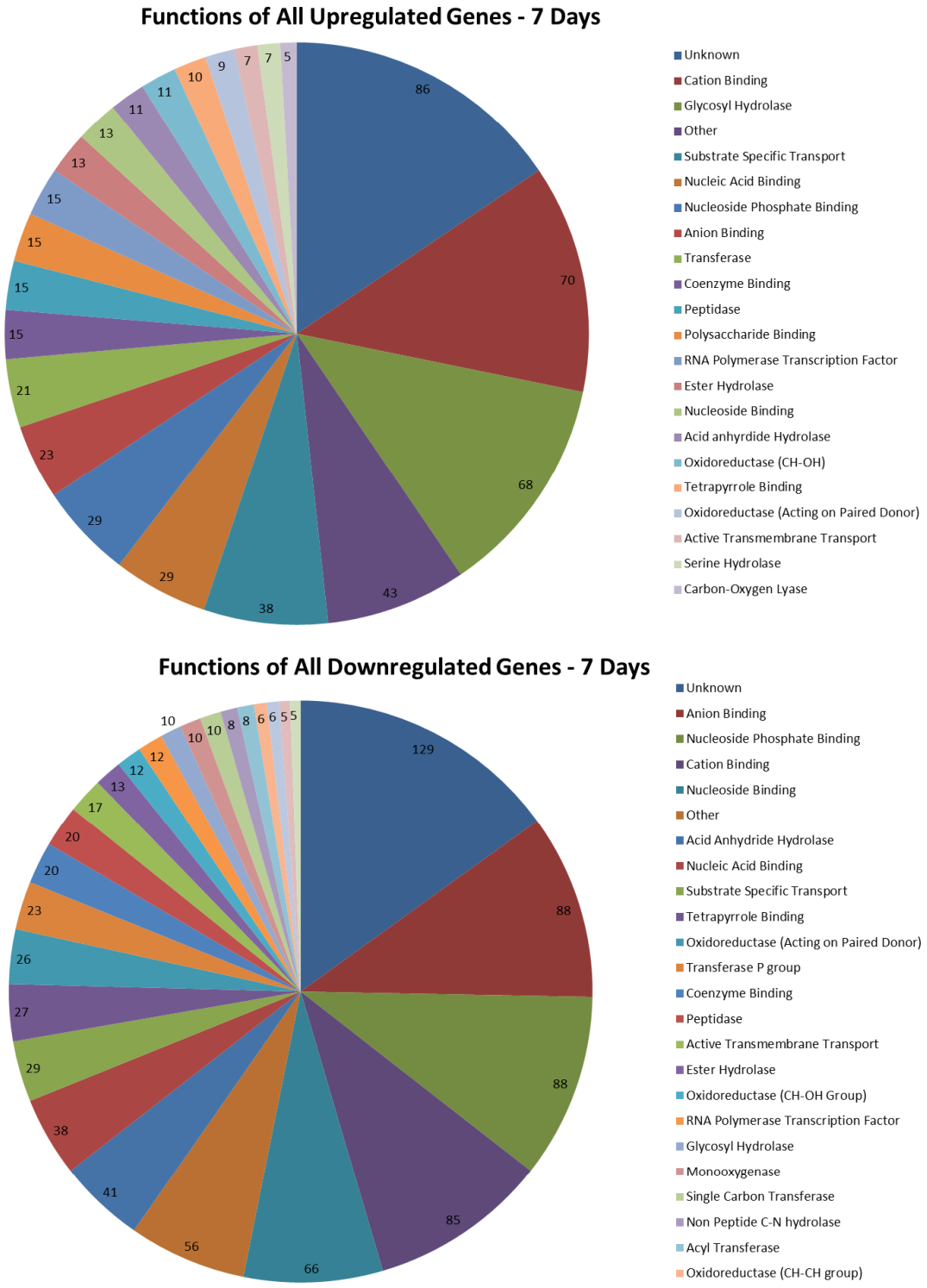


Figure 25: Functional categories of all differentially expressed genes at 7 DPI. BLAST, Interpro and GO searches implemented in BLAST2GO were used to construct functional categories for up-regulated (A) and down-regulated (B) genes in the 7 DPI dataset. The number of genes present in each functional group is indicated inside the pie slices. Unknown proteins and glycosyl hydrolases were again the most significantly up-regulated groups.

5.4.5 Overall Trends: *Trichoderma* Dataset

In order to identify all consistently up-regulated genes across the *T. virens* time points all samples were compared to the control and expression patterns analysed. MA and volcano plots show that a large number of genes remained detectable as differentially expressed across all datasets (**Figure 26**).

The functions of the identified genes confirmed general trends observed in the individual datasets. Glycoside hydrolases formed the most consistently up-regulated group, with 51 hydrolase encoding genes being expressed across the entire dataset. A large number of proteins supporting this polysaccharide degradation such as the acetyl xylan esterases and sugar transporters were also consistently up-regulated. Eight of the 20 transporters in this dataset were polysaccharide related. Genes of unknown function formed the second biggest group and as before, cation binding the third. It is interesting to note the large number of consistently up-regulated genes encoding 'other' proteins, which are comprised of functionalities with less than 5 representatives. This suggests that these groups are essential to the endophytic lifestyle and must be consistently maintained.

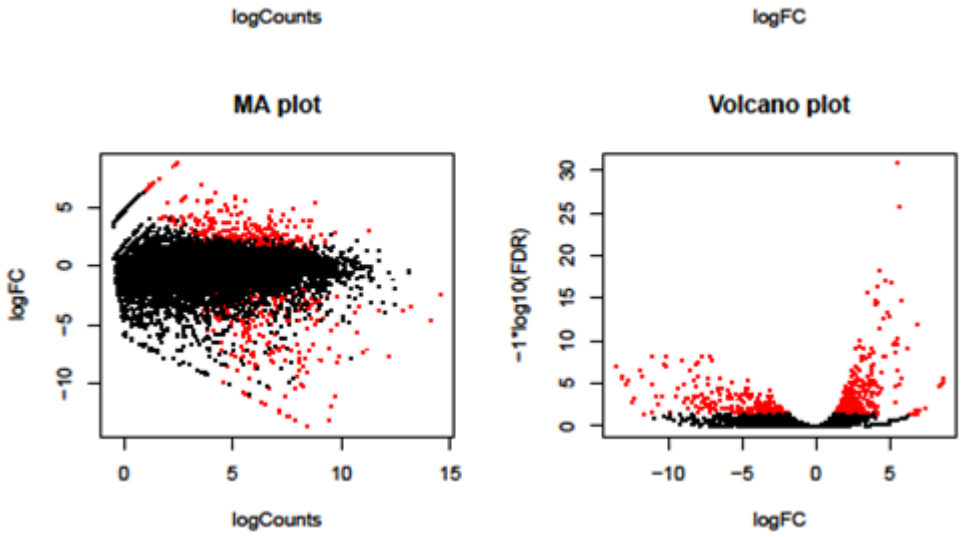


Figure 26: Volcano and MA plots for the comparison of all *T. virens* DPI datasetsMA plots show logCounts vs LogFC (Log fold change). LogFC indicates the log of the change in expression compared to the control T. virens. Log counts are the log of the read counts mapping to each gene. The most robust data are indicated by points with the highest logCounts, whereas the most differentially expressed is indicated by those values at the extreme top or bottom of the map (indicating down or up regulation respectively). Red dots indicate transcripts that have statistically significant differential expression. Volcano plots show the LogFC vs the –log10FDR (False discovery rate), points with more fold change and higher –log10FDR are more reliable. This graph clearly shows a large number of significantly differentially expressed genes were detected across all datasets compared to the control.

Genes up-regulated across the duration measured include TV_52152, the aflatoxin biosynthetic ketoreductase, which may suggest a continuing role for this toxin throughout the interaction. TV_47880, with a BLAST match to a trichothecene toxin biosynthetic enzyme was also present in this dataset. Many of the other volatile biosynthetic pathways, such as quinate and shikimate biosynthesis were also maintained across the interaction (**Figure 27**). Alkaloid metabolism was also well supported in this graph. Secondary metabolites may therefore be performing an important role in maintenance of cross communication between the host and fungal coloniser. Cluster analysis of this dataset showed similar results to other time points, with the most up-regulated cluster comprising a number of hydrolases and unknown proteins. The aflatoxin synthesis protein also clustered in this group, along with a steroid biosynthetic pathway protein.

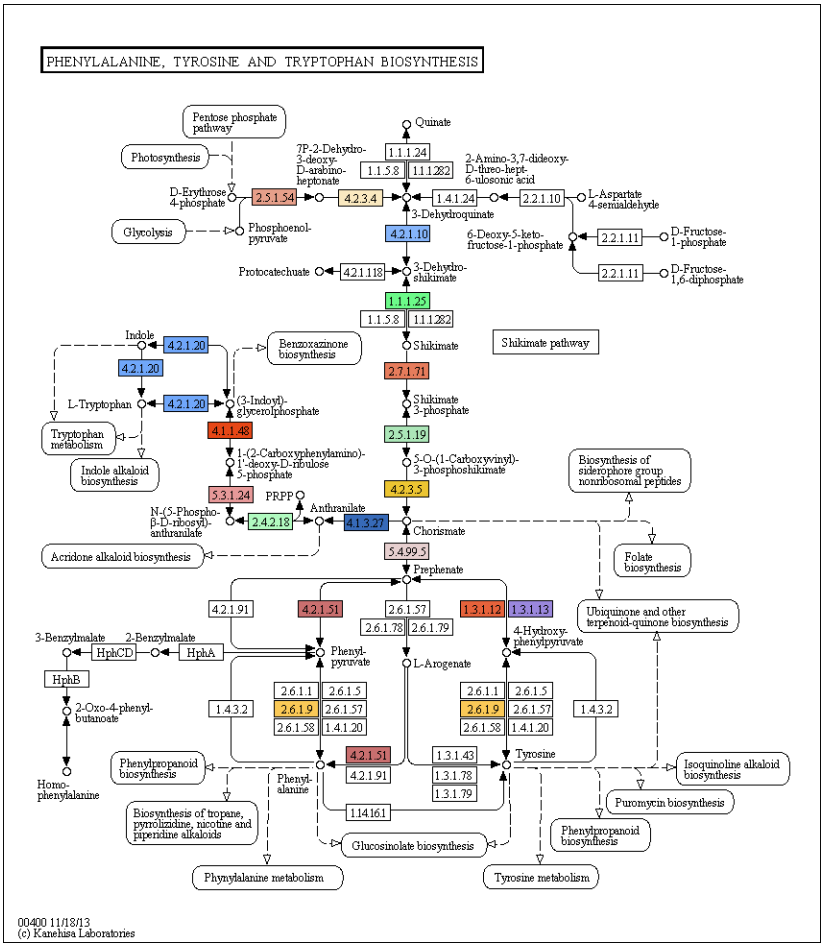


Figure 27: Up-regulated genes in the shikimate and related pathways across the entire interaction. The majority of the pathway has up-regulated genes during the interaction, supporting the role of volatiles, alkaloids and phenols during root colonisation. Plot generated in Blast2GO Kegg Analysis.

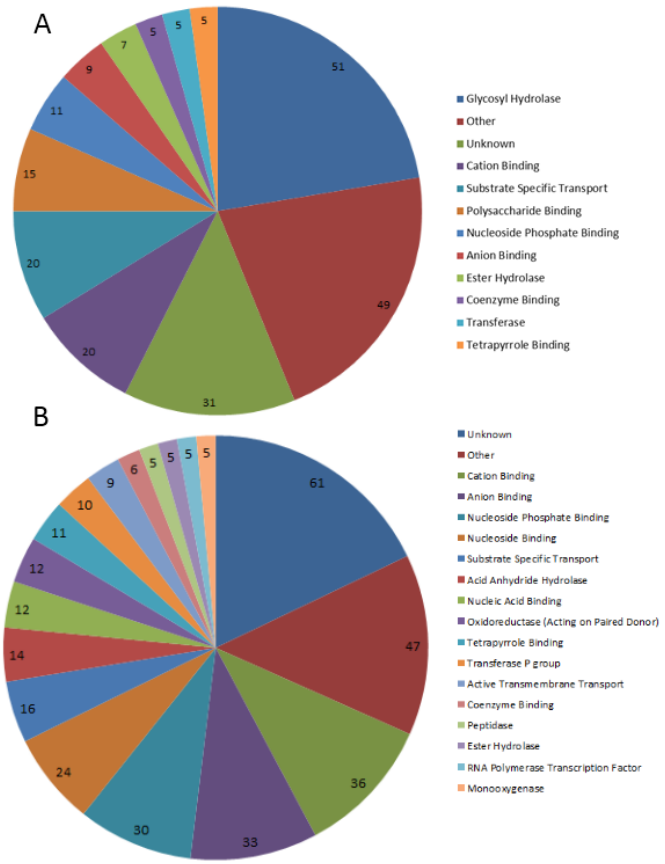


Figure 28: Functional categories of differentially expressed genes at across all DPI datasets.BLAST, Interpro and GO searches implemented in BLAST2GO were used to construct functional categories for up-regulated (A) and down-regulated (B) genes in the 7 DPI dataset. The number of genes present in each functional group is indicated inside the pie slices. Clearly the most consistently up-regulated group across all datasets is the glycoside hydrolases.

5.4.6 Transient Expression Analysis

In order to determine which proteins changed in each of the DPI samples, differential expression analysis was performed on samples from each of the different DPI. MA plots were constructed from these samples (**Figure 28**). The most notable observation was the similarities between the 3 and 5 DPI samples when compared to the 7 DPI *T. virens,* observable in both the MA plots and the heatplots (**Figure 30**). The 7 DPI dataset has both a larger number of different genes compared to the earlier time point and interestingly a number of genes expressed at the earlier time points appear to be switched off in the 7 DPI sample. This may indicate a changing transcriptional profile at longer time points, hinting that genes related to long term endophytism may be different to those required for initial colonisation; however more research would be needed to confirm this hypothesis. This would be consistent with a shift from overcoming the host-plant's immunity, to establishing a sustainable relationship, where the fungus can gain nutrition without damaging the host plant.

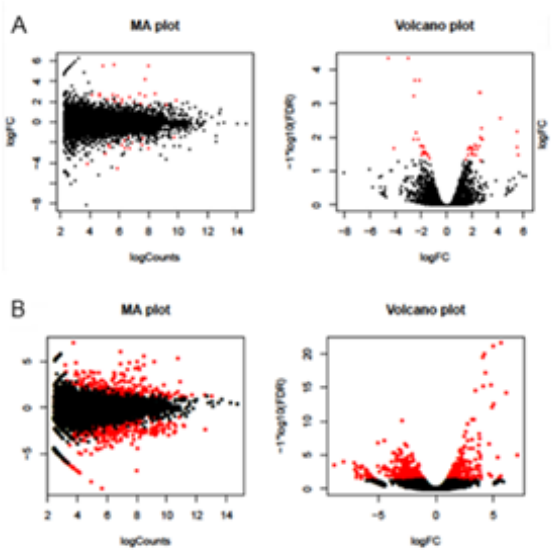


Figure 29: Volcano and MA plots for the comparison of 3 DPI to 5 DPI (A) and 5 DPI to 7 DPI (B)MA plots show logCounts vs LogFC (Log fold change). LogFC indicates the log of the change in expression compared to the control T. virens. Log counts are the log of the read counts mapping to each gene. Red dots indicate transcripts that have statistically significant differential expression. Note that 3 DPI and 5 DPI samples appear less different than the 5 and 7 DPI samples.

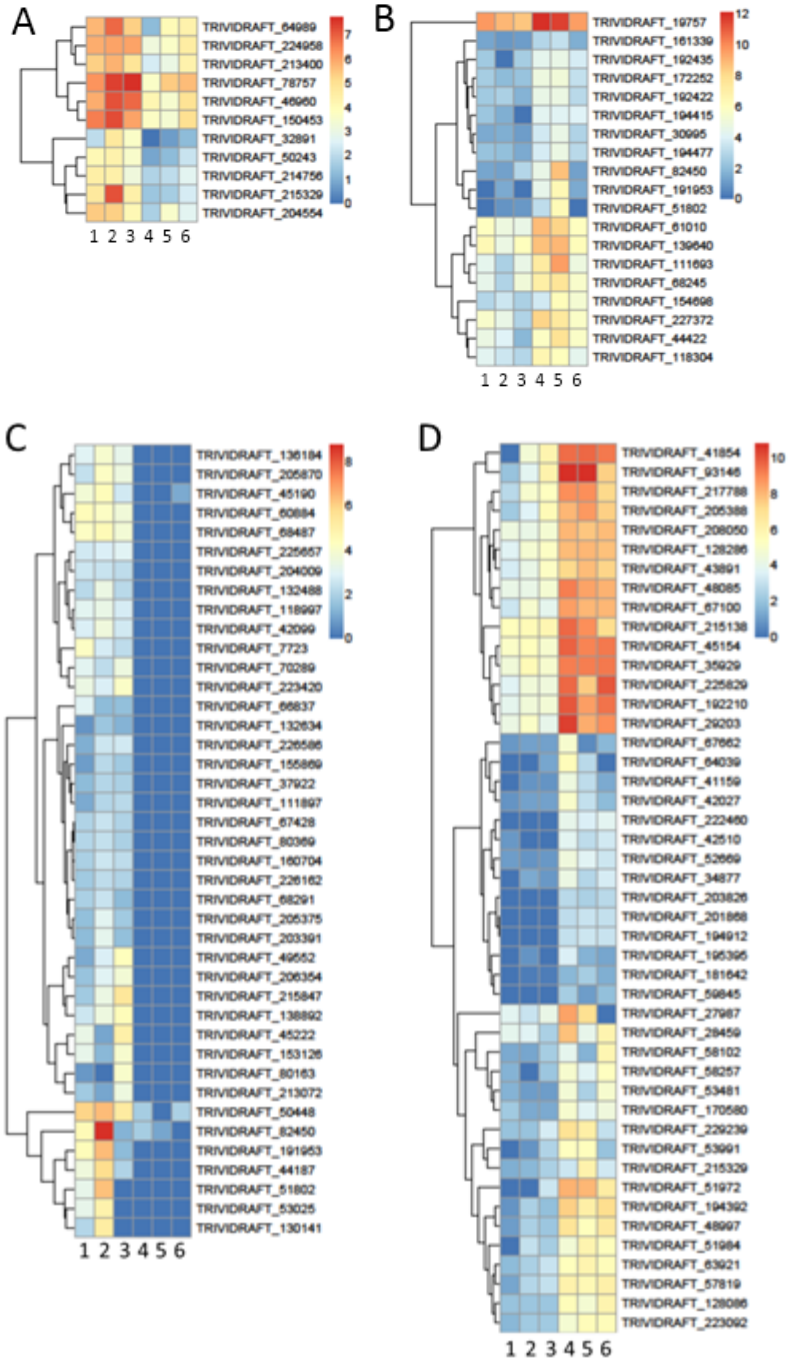


Figure 30: A clustered heat plot showing differential expression between *T. virens* at 3 vs 5 DPI and 5 vs 7 DPI.Heatplots were created from FPKM values derived from LogFC and count data. The top 50 most up-regulated genes in 3 DPI compared to 5 DPI are shown in **A** and the top 50 most up-regulated genes in 5 DPI compared to 3 DPI are shown in **B**. The top 50 most up-regulated at 5 DPI compared to 7 DPI (**C**) and top 50 most up-regulated (**D**) genes at 7 DPI compared to 5 DPI are shown. Each column represents one sample. Red represents the highest expression, blue represents the lowest. Very few genes were differentially expressed in these two datasets. TRIVIDRAFT is the alternate JGI identifier code from TV_.

5.4.7 Quality Control: Maize DPI dataset

The quality of the DPI maize data was high. Approximately 14 million reads mapped to maize in each interaction sample and approximately 20 million reads mapped to maize in the control samples. Dispersion was relatively low and BCV values were within acceptable parameters, albeit somewhat higher than the results for *T. virens*. This indicates that there was more variation between samples than in the fungal data.

Table 26: Dispersion and BCV for the maize Datasets.

Sample	Z. mays Reads	Dispersion	BCV
3 d 1	14203933	0.11244	0.3353
3 d 2	13958480		
3 d 3	14238010		
5 d 1	13547996	0.13543	0.368
5 d 2	12324364		
5 d 3	7158965		
7 d 1	7355148	0.08866	0.2978
7 d 2	7087685		
7 d 3	6638644		
3dM Cont	2870635		
5dM Cont	2946595		
7dM Cont	3162065		

The reads mapping to *Z.mays* in the DPI datasets are shown here. Dispersion and BCV values for this data indicate that the DPI datasets are less variable than the HPI datasets and thus are likely to provide more accurate differential expression data. The lack of replication in cDNA samples made it impossible to calculate Dispersion and BCV values for these datasets.

MA and volcano plots showed a large number of differentially expressed genes were identified in the 3 and 5 DPI datasets. The 7 DPI maize had a dramatically reduced number of differentially expressed genes compared to the other two datasets (**Figure 31**:).

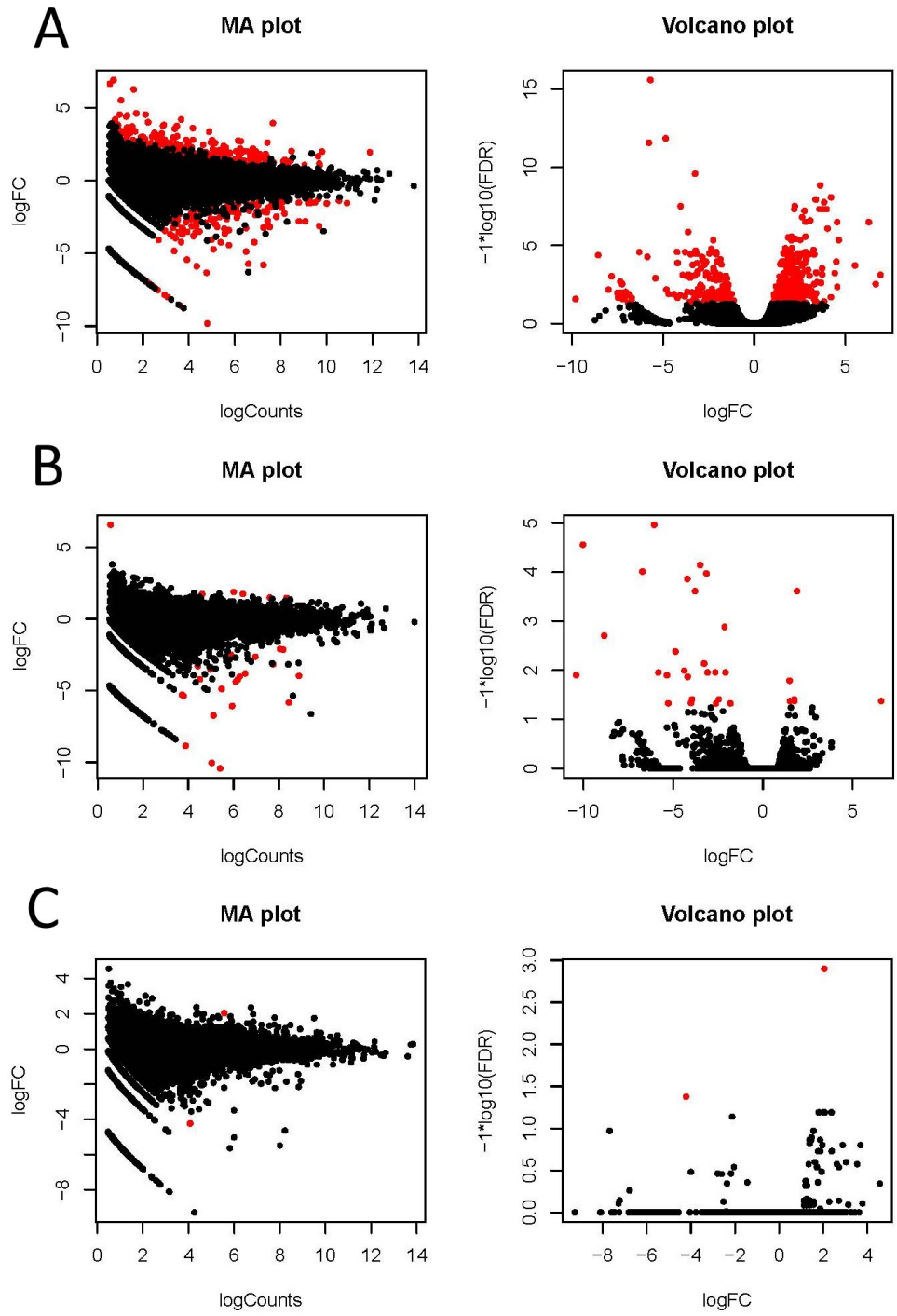


Figure 31: Volcano and MA plots *Zea mays* at 3 DPI (A), 5 DPI (B), and 7 DPI (C)MA plots show logCounts vs LogFC (Log fold change). LogFC indicates the log of the change in expression compared to the control T. virens. Log counts are the log of the read counts mapping to each gene. The most robust data are indicated by points with the highest logCounts, whereas the most differentially expressed are indicated by those values at the extreme top or bottom of the map (indicating down or up-regulation respectively). Red dots indicate transcripts that have statistically significant differential expression. Volcano plots show the LogFC vs the –log10FDR (False discovery rate), points with more fold change and higher –log10FDR are more reliable

5.4.8 Maize Three Days Post Inoculation

An analysis of cellular differential expression identified a number of transcriptional changes in functions likely to be related to plant defence, including stress, secondary metabolism, hormones and protein degradation (**Figure 32**). Three hundred and eighty genes were up-regulated and 557 genes were down-regulated in maize at 3 DPI. Ion and organic compound binding were the primary functional groups that were differentially expressed in both up and down-regulated time points (**Figure 33**).

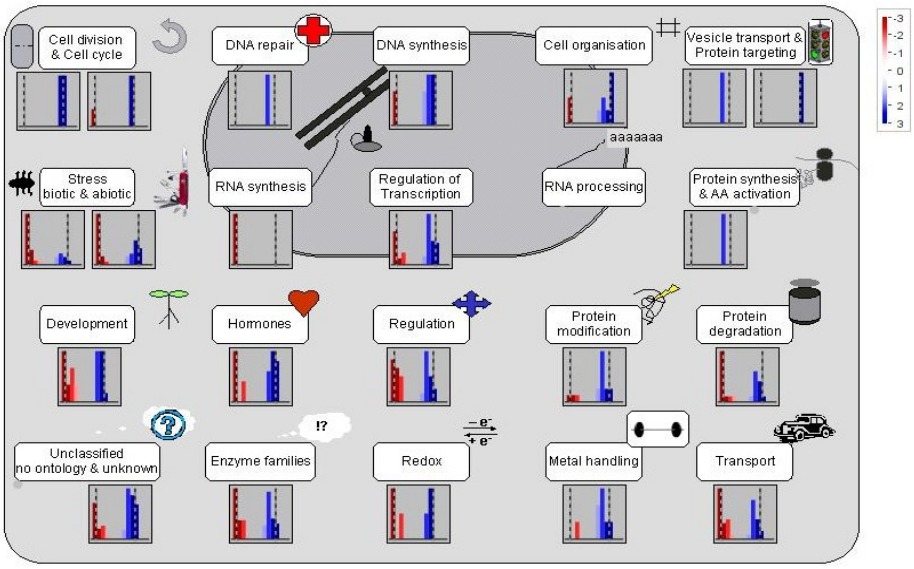


Figure 32: MapMan functional class map of differential expression in maize cellular processes at 3 DPI. Pathway maps were created in Mapman using the Zea mays genome release 09. The extent of differential expression is visualised based on LogFC data from EdgeR. Red indicates up-regulation, blue indicates down regulation.

Closer analysis of the stress pathways showed a majority of up-regulated genes were related to biotic stress or stress genes of unknown specific function. Down-regulated stress genes were predominantly in heat response pathways. Genes related to biotic stress encode proteins with functionalities including leucine rich repeat receptors (LRR receptors), WRKYGQK-domain containing transcription factors (WRKY proteins) and several cognate resistance proteins. Seven LRR receptors were identified in the dataset. LRR proteins are responsible for recognition of pathogenic MAMPs or effectors and induce local and systemic defence in plants (Boller & Felix, 2009). WRKY transcription factors are signalling proteins capable of instigating a range of plant responses through a variety of pathways such as calcium signalling and MAP kinase cascades (Rushton, Somssich, Ringler, & Shen, 2010). In defence signalling WRKY proteins are responsible for processing and amplification of signals from LRR proteins and often few WRKY proteins are required

to elicit a large scale response (e.g. 4 WRKY genes in *Medicago trunculata* have wide ranging effects from lignin deposition to systemic defence) (Pandey & Somssich, 2009). WAK (Wall associated kinase) proteins are responsible for extracellular perception and signalling responses to the environment and can mediate the response to a pathogen (Kanneganti & Gupta, 2008). The presence of LRR, WAK and WRKY proteins is thus unsurprising but is an important demonstration of a classical defence signalling pathway being activated in this interaction. It was notable that some LRR proteins, such as GRMZM2G066274, were down-regulated at 3 DPI, potentially indicating interference by *T. virens* in maize defence.

Table 27: The 50 most up-regulated genes in *Zea mays* at 3 DPI

Seq. Identifier	Seq. Description	LogFC
GRMZM2G136372	pathogenesis-related protein 5	-12.07970152
TPS6	bicyclic sesquiterpene synthesis	-11.81553673
GRMZM2G028306	terpene synthase 5	-10.91120045
GRMZM2G448458	histone h2a	-10.16288462
GRMZM2G051720	terminal uridylyltransferase 7-like	-9.792071903
GRMZM2G154828	cytochrome p450 superfamily protein	-9.749070205
GRMZM2G151227	chalcone synthase	-9.560185745
GRMZM2G173965	mitogen-activated protein kinase kinase kinase a-like	-9.382013786
GRMZM5G893953	unknown	-9.080868798
GRMZM2G070011	cysteine proteinase ep-b 1-like	-9.047977917
GRMZM2G333274	galactoside 2-alpha-l-fucosyltransferase-like	-8.915596912
AC190772.4	manganese binding protein	-8.770908794
GRMZM2G050321	salicylate o-methyltransferase-like	-8.759272846
GRMZM2G017223	acid phosphatase 1-like	-8.686531229
GRMZM2G430942	acidic endochitinase-like	-8.67140398
AC198414.2	unknown	-8.663038818
AC214817.3	protein kinase	-8.471524293
NIP1-1	aquaporin	-8.456775456
GRMZM2G033665	MLO-like protein 1	-8.417022004
GRMZM2G448672	probable receptor-like protein kinase at1g67000-like	-8.37731903
GRMZM2G084779	potassium transporter 5-like	-8.3760741
GRMZM2G475014	NAC domain transcription factor protein	-8.365456792
GRMZM2G021277	aromatic-l-amino-acid decarboxylase-like	-8.342976629
GRMZM2G343972	ring zinc finger and VWF domain family protein	-8.266756267
GRMZM2G021388	aromatic-l-amino-acid decarboxylase-like	-8.252671793
GRMZM2G453672	LRR receptor-like serine threonine-protein kinase	-8.249473343
GRMZM2G169261	omega-6 lipidty acid endoplasmic reticulum isozyme 2	-8.208439013
GRMZM2G036365	aspartic proteinase nepenthesin-1-like	-8.179863629

GRMZM2G173742	retrotransposon expressed	-8.124698997
GRMZM2G082504	hypothetical protein ZEAMMB73_207934	-8.123945904
GRMZM2G169584	NBS-LRR-like protein	-8.088159181
GRMZM2G161245	malate chloroplastic-like	-8.070266167
GRMZM2G114588	short chain dehydrogenase	-8.038080622
GRMZM2G010702	hypothetical protein	-8.018152665
GRMZM2G052796	glutaredoxin-like protein	-7.992784968
GRMZM2G453311	salutaridine reductase-like	-7.884163347
GRMZM2G006853	pathogenesis-related protein 5	-7.809391698
GRMZM2G328593	nitric oxide synthase-interacting	-7.804569337
GRMZM2G357834	senescence associated gene	-7.792555132
GRMZM2G420280	scarecrow-like protein 18-like	-7.731262518
GRMZM2G064885	unknown	-7.714031795
GRMZM2G386987	hypothetical protein ZEAMMB73_654185	-7.661784207
GRMZM2G391815	pleiotropic drug resistance protein 5-like	-7.631932285
GRMZM2G175480	transcription factor bhlh041-like	-7.599144341
GRMZM2G106560	probable WRKY transcription factor 75-like	-7.590466227
GRMZM2G467520	hypothetical protein ZEAMMB73_412982	-7.583783349
GRMZM2G406643	ubiquitin-60s ribosomal protein l40-like	-7.583187775
CAT3	circadian rhythm regulator	-7.580191798
GRMZM2G094375	laccase 25 oxidoreductase	-7.570573086

The 50 most highly up-regulated genes in maize at 3 DPI and their descriptions as identified by BLAST searches. LogFC is the log fold change as compared to the control maize at 3 DPI – LFC is shown as negative as it reads 'Change in control compared to the sample, i.e. for TPS6 the control has -12.079 expression compared to the weighted average of the samples.

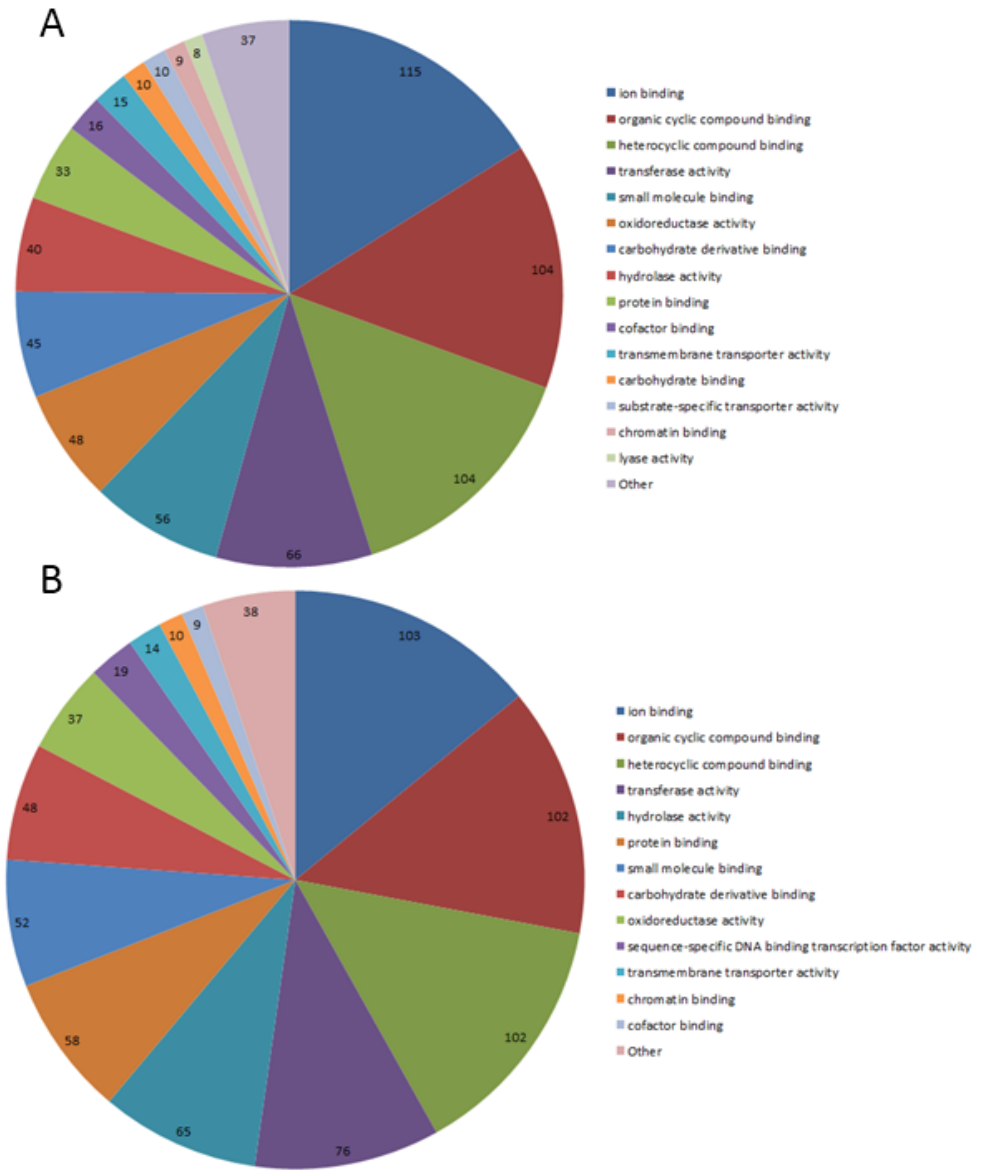


Figure 33: Functional categories of differentially expressed genes in *Zea mays* at 3 DPI.BLAST, Interpro and GO searches implemented in BLAST2GO were used to construct functional categories for up-regulated (A) and down-regulated (B) genes in the 3 DPI dataset. The number of genes present in each functional group is indicated inside the pie slices. It is difficult to see a clear pattern in functionality in the maize dataset.

Genes that comprise part of both lignin biosynthesis and lignin deposition are up-regulated, and these are often part of cell wall modification responses involved with defence against pathogens (Bhattacharya, Sood, & Citovsky, 2010; Gilbert, 2010). Lignin pathways are supported by the presence of several lignin related genes that are up-regulated in this experiment (**Figure 34**). They are also related to plant signalling, response to predation and can act directly as toxic agents upon invading pathogens (Lattanzio, Lattanzio, & Cardinali, 2006). Hormone regulated pathways were altered to a lesser extent, with two proteins each in the ethylene and salicylic acid pathways being

up-regulated. Ethylene is usually responsible for plant growth regulation and may reflect the early developmental stage of the plant. Salicylic acid (SA) is a phenolic compound derivative and is a major facilitator of plant responses to a broad range of pathogens, to the extent that SA deficient mutants are severely immune-compromised (Lu, 2009). The gibberellic acid (GA) pathway has several repressed proteins and GA is signalling is antagonistic to several other defence pathways (particularly JA). *T. virens* expresses GA related enzymes at 3 DPI (Yang et al., 2012).

Table 28: The 50 most down-regulated genes in *Zea mays* at 3 DPI

Seq. Identifier	Seq. Description	LogFC
GRMZM2G473960	late embryogenesis abundant group 1	8.045755
GRMZM2G425629	late embryogenesis abundant group 3-like	7.381538
GRMZM2G448627	late embryogenesis abundant group 1	7.294616
GRMZM2G065227	uncharacterised loc101212329	7.294616
GRMZM2G143530	FAD-binding berberine family protein	7.103283
GRMZM2G075524	hypothetical protein ZEAMMB73_861375	6.882638
GRMZM2G121937	unknown protein	6.882638
GRMZM2G132371	kinesin-related	6.622047
GRMZM2G014844	glycosyl hydrolase	6.622047
MGL3	late embryogenesis abundant group 3-like	6.085871
GRMZM2G331701	class IV heat shock protein precursor	5.801376
GRMZM5G897067	mRNA	5.586298
GRMZM2G121928	retrotransposon	5.581931
GRMZM2G140293	hypothetical protein ZEAMMB73_473940	5.537206
GRMZM2G169943	aldose reductase	5.310846
GRMZM2G154747	plasma membrane associated protein	5.292412
GRMZM2G428040	mediator of RNA polymerase II transcription subunit 26	5.139555
DHN1	Dehydrin (Late embrogenesis abundant)	5.138765
GRMZM2G103771	stress-inducible membrane pore protein	5.097295
GRMZM2G047941	DUF231 domain containing family protein	4.869344
GRMZM2G146395	ferredoxin- chloroplastic-like	4.809766
GRMZM2G071101	protein transparent testa 1-like	4.802509
GRMZM2G179462	membrane potential modulator	4.560125
GRMZM2G102138	retrotransposon	4.558473
GRMZM2G063287	Late embryogenesis protein	4.520949
GRMZM2G450125	glycosyl hydrolase family 14	4.516599
GRMZM2G162659	late embryogenesis abundant protein	4.416651
GRMZM2G010494	chloroplastic-like	4.387386
GRMZM2G127162	hypothetical protein ZEAMMB73_232413	4.36887
GRMZM5G805685	Ap2/B3 transcription factor	4.296206
GRMZM2G052105	LYR motif-containing protein at3g19508-like	4.288682

GRMZM2G165726	unknown protein	4.168796
GRMZM2G166776	hypothetical protein ZEAMMB73_955786	4.13271
GRMZM2G401983	lipoprotein A	4.125755
GRMZM2G027052	Auxin canalisation domain	4.11792
GRMZM2G026911	aspartyl protease	4.101062
GRMZM2G016558	ankyrin repeat-containing protein at3g12360-like	4.055606
GRMZM2G066274	LRR protein	4.014042
GRMZM2G129361	TPA: hypothetical protein ZEAMMB73_521774	4.012602
GRMZM2G092968	protodermal factor 1	3.991692
GRMZM2G150215	regulatory protein	3.990959
GRMZM2G118770	NADP-dependent malic enzyme	3.950854
GRMZM2G056236	lipoprotein A	3.93392
GRMZM2G156126	alpha beta-hydrolases superfamily protein	3.927989
GRMZM2G067600	uncharacterised loc101215398	3.911004
GRMZM5G828487	protein iq-domain 14-like isoform x1	3.899948
GRMZM2G124963	alanine aminotransferase	3.893546
GRMZM2G042013	hypothetical protein ZEAMMB73_263283	3.88795
GRMZM2G081251	unknown protein	3.886842

The 50 most highly down-regulated genes in maize at 3 DPI and their descriptions as identified by BLAST searches. *LogFC is the log fold change as compared to the control maize at 3 d. LogFC is positive as it is up-regulated in the Control compare to the samples. i.e. the GRMZM2G081251 gene is 3.88 up-regulated in the control compared to experimental samples.

Figure 34 shows a pathway map (genes are linked to known maize pathways using Mapman referencing the B73 maize genome) of receptor and signalling protein regulation in maize at 3 DPI. Over 100 regulatory genes were differentially expressed at this time point, indicating significant transcriptional regulation occurring within the plant. Thirty of these genes encode proteins of possible relevance to defence, including 11 NBS-LRR repeat proteins (five up and six down regulated), nine DUF proteins (eight up and one down regulated), four S-locus receptors (three up and one down regulated), four cytoplasmic receptor kinases (all down regulated)and two up-regulated WAK receptors. LRR proteins are the canonical defence receptor protein, responsible for gene-for-gene type defence (McHale et al., 2006). The low number of differentially expressed LRR proteins is surprising, suggesting that *T. virens* elicitors are only being detected by a small number of these proteins. Masking of *T. virens* from plant receptors may explain the formation of a mutualistic interaction between *T. virens* and maize, as the detection of numerous elicitors would suggest a large scale plant immune response. However, in other plant-fungal systems even a single elicitor can be enough to initiate a host defence response (Jia, McAdams, Bryan, Hershey, & Valent, 2000; Van den Ackerveken, Van Kan, & De Wit, 1992). This has been demonstrated in tomato, where the Cf-4 and Cf-9 genes confer resistance by detecting Avr4 and Avr 9 respectively (Thomas

et al., 1997). Calcium signalling is also differentially expressed here, and has been implicated as a growth promoting and early signalling element in endophytes of *Arabidopsis* (Vadassery et al., 2009). This may indicate that fungal manipulation of plant signalling is mitigating the host response, despite the detection of several *Trichoderma* elicitors by the host plant's LRR proteins.

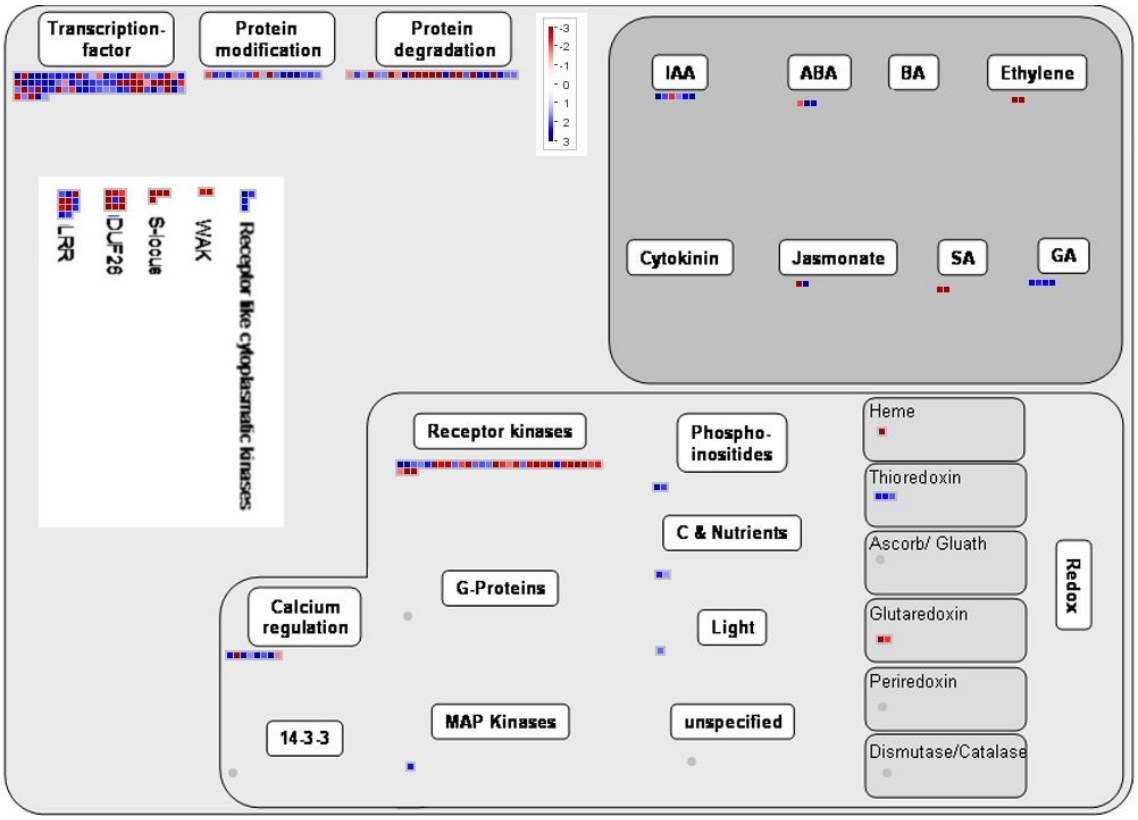


Figure 34: A pathway mapping of differential expression patterns for receptor and signalling proteins in maize at 3 DPI. Mappings were created in MAPMAN using the Zm_Genome_Release_09 and differential expression data from EdgeR. Up-regulated genes are visualised in red, down-regulated genes in blue. LRR and WAK receptors are central to plant defence signalling and both are represented in the dataset. Down-regulated LRR receptors may be undergoing active repression by fungal effector proteins. Receptor kinases are likely to function in enhancing the signalling response from LRR or WAK type proteins. Several hormone pathways, also appear to be undergoing transcriptional regulation, although no strong pattern is observed, even amongst defence related hormonal pathways such as salicylic acid.

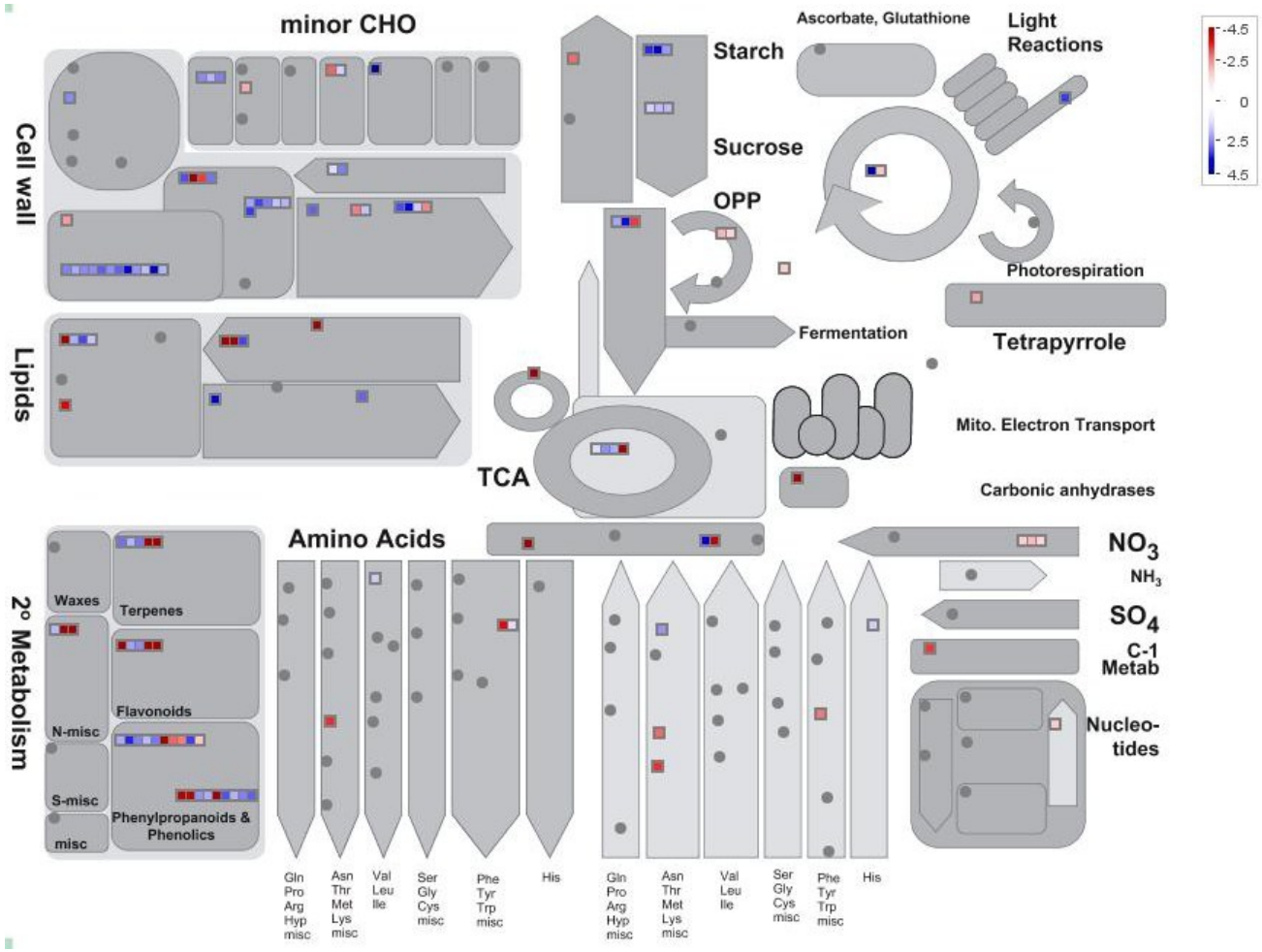


Figure 35: A pathway mapping of metabolic regulation in *Zea mays* at 3 DPI.Mappings were created in MAPMAN using the Zm_Genome_Release_09 and differential expression data from EdgeR. Up-regulated genes are visualised in red, down-regulated genes in blue. Cell wall pathways appear to be majorly down-regulated at 3 DPI, possibly as a result of T. virens interference with maize cell regulation. This is also surprising as the large amount of CWDEs produced by T. virens would have suggested an increase in maize cell wall production. The other major regulation alterations occur in phenylpropanoids, phenolics and terpenes, all of which are associated with highly bioactive compounds.

The degradation and metabolism of plant cell wall via lytic enzymes and associated transport molecules appeared to be well supported in *T. virens* (see **section 5.4.2.3**). These processes are supported in the plant transcriptome, visualised in **Figure 35** and **Figure 36**. Cell wall related functions are differentially expressed in maize at 3 DPI, as well as lignin, phenol and lipid pathways. These are all related to plant remodelling of the cell wall in order to fix damage to the cell wall, with lignification and callose deposition being primary responses to cell damage by invaders (Ellinger et al., 2013; Luna et al., 2011; Xu et al., 2011). Cell wall lignification is modulated by jasmonic acid and oxidative signalling, the first of which is differentially expressed in this dataset (Denness et al., 2011). Callose deposition is regulated by both SA and JA in *Arabidopsis*, both of which have limited differential expression here (Yi, Shiras, Moon, Lee, & Kwon, 2014). The differential expression of plant cell wall related and regulatory genes provides further support for the importance of cell wall degradation on *T. virens* root colonisation.

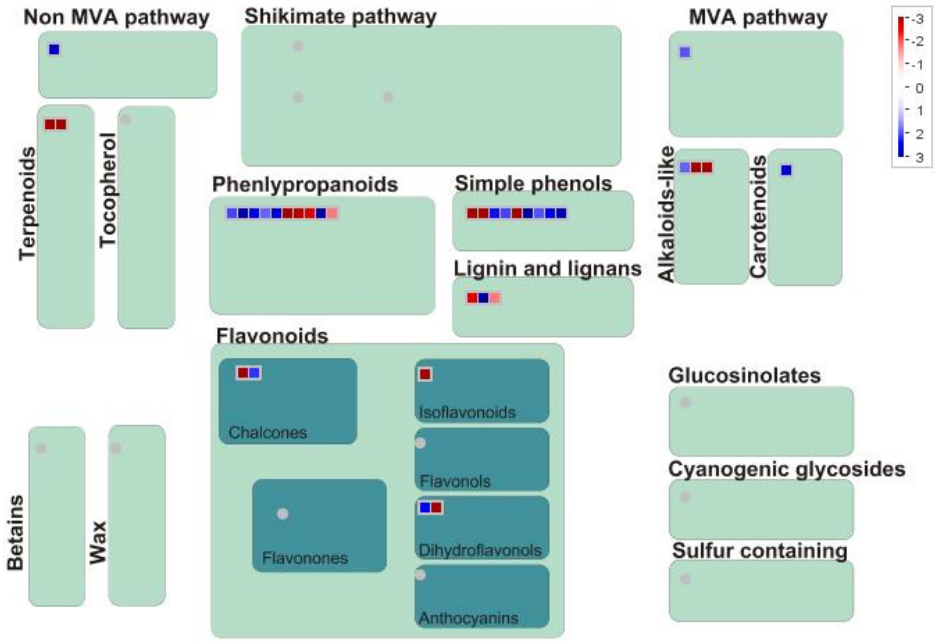


Figure 36: A MapMan pathway mapping of secondary metabolite regulation in *Zea mays* at 3 DPI.Mappings were created in MAPMAN using the Zm_Genome_Release_09 and differential expression data from EdgeR. Up-regulated genes are visualised in red, down-regulated genes in blue. Phenolics and phenylpropanoids are again undergoing major transcriptional changes. Also of interest is the regulation of lignins, these molecules are part of a common plant response to cell wall degradation, in which lignin is used to 'shore up' the plant cell wall after damage

5.4.9 Maize Five Days Post Inoculation

A large reduction in the number of differentially expressed genes was observed in the 5 d samples. Only 109 total genes were differentially expressed, with 88 up-regulated and 21 down-regulated genes being identified. The reduced numbers of differentially expressed genes may suggest a stabilization of the interaction over longer periods of time. Notable pathogenic response genes at 5 DPI include 4 LRR proteins, 9 peroxidases and perhaps most importantly a xylanase inhibitor (GRMZM2G133781). Xylanase inhibition would be an obvious response to the large scale up-regulation of cell wall degradation seen in the *T.* virens transcriptome, although the expression of a single xylanase inhibitor is unlikely to significantly affect *T. virens* given the scale of its lytic enzyme production. The lack of inhibitors expressed may indicate successful repression of plant countermeasures by *T. virens*. Peroxidases are responsible for the production of plant oxidative bursts, which are the initial line of plant defence upon detection of an invader (Daudi et al., 2012; O'Brien et al., 2012). The presence of NBS-LRR proteins at this time point may indicate the continued detection of the fungus by the plant, possibly causing this response. A single chitinase (GRMZM2G051943) is also up-regulated in this dataset and is an obvious response to fungal colonisation.

Table 29: The 50 most up-regulated genes in *Zea mays* at 5 DPI

Seq. Identifier	Seq. Description	LogFC
GRMZM2G156257	peroxidase 2	-11.0196
GRMZM2G030772	hypothetical protein ZEAMMB73_540056	-9.6807
GRMZM2G105935	root cap protein 1	-8.97314
GRMZM2G049852	mate efflux family protein 9-like	-8.94317
GRMZM2G027976	transcription factor RF2B-like	-8.91801
GRMZM2G028731	proline-rich receptor-like protein kinase perk8-like	-8.88995
GRMZM2G125196	alcohol dehydrogenase superfamily protein	-8.54311
GRMZM2G010372	protein walls are thin 1-like	-8.54035
GRMZM2G008316	glycosyltransferase-like domain-containing protein 2-like	-8.36429
GRMZM2G476523	thaumatin-like	-8.31668
GRMZM2G010356	terpene synthase 7	-8.25321
GRMZM2G009818	leucine-rich repeat receptor-like protein kinase pxl2-like	-7.18194
GRMZM2G111998	probable beta-D-xylosidase 2-like	-7.05228
GRMZM2G027167	cortical cell-delineating protein precursor	-7.02162
GRMZM2G477685	cortical cell-delineating protein precursor	-6.57043
GRMZM2G012904	hypothetical protein precursor	-6.56924
AC234161.1	unknown protein	-6.43397
GRMZM2G097349	homeobox-leucine zipper protein HAT7	-6.40747

TPS6	bicyclic sesquiterpene synthesis	-6.39973
GRMZM2G170829	hypothetical protein ZEAMMB73_814471	-6.24391
GRMZM2G436299	cellulose synthase-like protein d3	-5.95744
GRMZM2G093622	germin-like protein 12-2-like isoform x1	-5.84146
GRMZM2G410338	cortical cell-delineating protein precursor	-5.72609
GRMZM2G355098	protein kinase superfamily protein	-5.62243
GRMZM2G037156	peroxidase 9-like	-5.5857
GRMZM2G170017	salutaridine reductase-like	-5.3803
GRMZM2G410175	class III peroxidase	-5.35133
GRMZM2G036708	beta-cyanoalanine synthase	-5.32975
GRMZM2G097141	hypothetical protein ZEAMMB73_931355	-5.31084
GRMZM2G088765	peroxidase 15-like	-5.26894
GRMZM2G177445	probable serine threonine-protein kinase at5g41260-like	-4.99103
GRMZM2G403007	non-specific lipid-transfer protein 2-like	-4.93206
GRMZM2G168603	leucine-rich repeat receptor-like protein kinase PXL2-like	-4.92746
GRMZM2G448458	histone h2a	-4.85007
GRMZM2G027217	class III secretory plant peroxidase family protein	-4.83472
GRMZM2G169033	laccase family protein	-4.83236
GRMZM2G154828	cytochrome p450 superfamily protein	-4.75981
GRMZM2G028010	probable FAD synthase-like	-4.66844
GRMZM5G826700	pistil-specific extensin-like	-4.66835
GRMZM5G804575	nodulin 21 family protein	-4.65121
GRMZM2G157298	germin-like protein 12-4-like	-4.59777
GRMZM2G477697	cortical cell-delineating protein precursor	-4.57255
GRMZM2G022442	mannose acetylglucosaminyltransferase	-4.50069
GRMZM2G021427	beta-expansin 1a precursor	-4.49973
GRMZM2G149714	hypothetical protein ZEAMMB73_378483	-4.47853
GRMZM2G162461	phosphatidylinositol phosphatidylcholine transfer protein	-4.44786
H3C2	histone	-4.36657
GRMZM2G081529	unknown protein	-4.36487
GRMZM2G302171	TPA: hypothetical protein ZEAMMB73_090961	-4.34996

The 50 most highly up-regulated genes in maize at 5 DPI and their descriptions as identified by BLAST searches. * LogFC is the log fold change as compared to the control maize at 5 DPI.

Mapman analysis of the maize transcripts at 5 DPI further supported the activities discussed above (**Figure 37**). Peroxidases and related genes formed the major group of differentially expressed proteins, enhancing the likelihood of oxidative-burst like responses occurring at this time point. Cell-wall construction and secondary metabolites also feature at this time point, which was expected due to the high regulation of *T. virens* lytic enzymes across the time scale of the interaction. Biotic and abiotic stress related proteins were also up-regulated, which was again expected in the host plant during the early stages of microbial invasion. The most unusual

observation at this time point is the lack of a clear trend in the maize response to invasion by *Trichoderma*. Only peroxidase activity and cell wall modification were identifiable as clear elements that may remove or limit the growth of the fungus. The lack of a coordinated maize response may provide further evidence for the ability of the fungus to disrupt the ability of maize to respond to the invasion at 5 DPI.

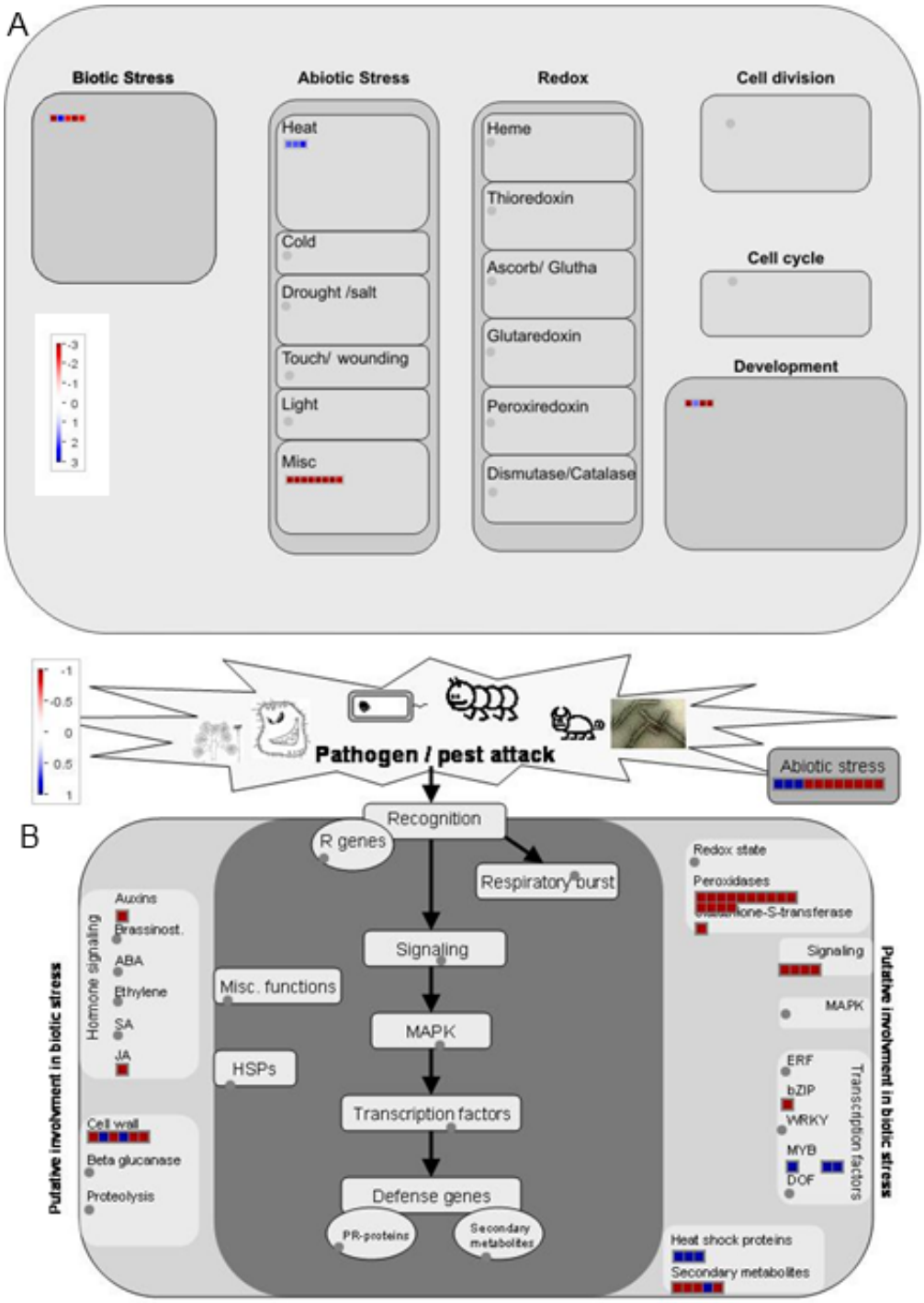


Figure 37: MapMan pathway mappings of maize differential expression at 5 DPI Cellular Response Overview (**A**) and Pathogen Related Genes (**B**).Mappings were created in MAPMAN using the Zm_Genome_Release_09 and differential expression data from EdgeR. Up-regulated genes are visualised in red, down-regulated genes in blue. Significantly fewer regulation changes are observed compared to maize at 3 DPI. A number of peroxidases were up-regulated at 5 DPI, as well as several secondary metabolites.

5.4.10 Maize Seven Days Post Inoculation

Only 22 differentially expressed genes were identified in maize at 7 d and the majority of these were down-regulated. Several of these proteins were of interesting function. GRMZM2G030252 was amongst the 4 up-regulated genes at 7 DPI and it encodes for walls are thin 1 (WAT1). This protein is responsible for cell wall regulation in plant defence and supports SA defence signalling pathways (Nicolas Denancé, 2012). Cell wall regulation thus continues to be a consistent trend in the interaction. Other up-regulated genes encode an unknown protein, DNA topoisomerase and BX1. BX1 is responsible for defence against insect herbivory and has also shown to regulate the response to fungal pathogens in maize (Ahmad et al., 2011). Again the limited scope of plant responses may be indicative of the fungus' ability to systematically avoid host immune responses.

Table 30: All differentially expressed genes in *Zea mays* at 7 DPI

Seq. Identifier	Seq. Description	logFC
GRMZM2G092804	**unknown**	-2.74266
GRMZM2G021270	**DNA topoisomerase 2-like**	-2.60743
GRMZM2G030252	**WAT1-related protein at3g30340-like**	-2.55352
BX1	DIMBOA regulation (leaf feeding resistance)	-2.22346
GRMZM2G168898	hemoglobin 2	-2.0968
GRMZM5G879116	SEC14 cytosolic factor-like	1.702388
GRMZM2G128682	pectinesterase pectinesterase inhibitor 41	1.825478
GRMZM2G130904	peroxidase n-like	1.988656
GRMZM2G091478	ABC transporter a family member 7-like	2.132437
GRMZM2G010280	high affinity nitrate transporter	2.468647
GRMZM2G018716	beta- insoluble isoenzyme 7	2.632308
GRMZM2G136567	auxin-induced protein 5NG4-like	2.703029
GRMZM2G475380	oxoglutarate 3-dioxygenase	2.903437
GRMZM2G022699	hypothetical protein ZEAMMB73_861720	2.93507
GRMZM2G116902	peroxidase n-like	2.959422
GRMZM2G143883	long-chain-alcohol oxidase FAO1-like	3.114146
GRMZM2G064091	nitrate transporter -like	3.645118
GRMZM2G131421	early nodulin 93	4.325473
GRMZM2G034421	WRKY55 - WRKY and zinc finger domains	4.398632
GRMZM2G169149	WRKY DNA-binding domain superfamily protein	4.41795
GRMZM2G316362	acyl-	4.573891
GRMZM2G180918	hypothetical protein ZEAMMB73_010704	7.582726
GRMZM2G078441	vicilin-like antimicrobial peptides 2-2-like	7.713315

All up (**bold)** and down-regulated maize genes at 7 DPI and their descriptions as identified by BLAST searches. *LogFC is the log fold change as compared to the control maize at 5 DPI.

Down-regulated genes included 18 proteins. Two WRKY proteins were present in this dataset and may be down-regulated either due to *Trichoderma* effectors or because they are negative regulators of immune processes. GRMZM2G136567, an auxin induced protein was down-regulated in this dataset and may potentially link to WAT1 activity. Other down-regulated proteins had no obvious link to defensive responses.

5.5 Integrated Analysis of Maize and *T. virens* Results

The interaction between *T. virens* and maize during root colonisation is intricate and involves complex pathways. Evidence collected in this study suggests that two major pathways (cell wall polysaccharide degradation and secondary metabolites) play a role in initial root colonisation, alongside a number of less well supported groups and possible effectors. Polysaccharide degradation forms the most consistent and obvious response in *Trichoderma*. Volatile compound biosynthesis, particularly in relation to hormone production and phenolic compounds appears to play a secondary, but well supported role. Functional groupings such as toxins, peroxidases and transporters appear to support the functionality of other groups or aid in overcoming plant immunity.

Effector proteins were hypothesised to play a major role in root colonisation, via secretion of small bioactive proteins that modulate host plant function. Transcriptome analysis identified a large number of unknown proteins that were up-regulated in all the DPI datasets. A relatively large number of unknown proteins were detected in all DPI datasets (average of 83). The lack of identifiable motifs in these proteins, even after BLAST, Interpro and manual searches makes it difficult to comment on potential function, except to suggest that it is highly likely that at least one of these is an effector protein. Small secreted cysteine rich proteins were, however readily identified in both the bioinformatics and transcriptome analyses, with 24% crossover between the datasets. The majority of these were hydrolase enzymes or proteases of various types. These results suggest that, while still potentially a major influence on root communication, other pathways are of equal significance in the formation of endophytic relationships particularly hydrolytic enzymes.

Although appressoria and haustoria form possible physical methods of plant entry, endophytes have other methods of cell wall penetration. Glycoside hydrolases provide a clear mechanism for both host penetration and nutrition acquisition in *T. virens*-maize interactions. **Figure 38** shows the structure of plant cell walls, with cellulose, pectin and hemicellulose comprising the majority of the

plant cell wall (Smith, 2001). These are composed of linked monomers of xylose, mannose and glucose, in the case of cellulose and hemicellulose, and various galacturonan polysaccharides in the case of pectin (Gilbert, 2010). These polysaccharides may also be modified by various phenolic or saccharide groups which may link *Trichoderma* secondary metabolite degradation pathways to cell wall degradation processes as well.

Table 31 shows the diversity of function of the hydrolase groups up-regulated during this interaction. Xylanase and cellulose degrading enzymes dominate the functionalities, however almost all plant polysaccharides can be degraded using these enzymes.

Table 31: Glycoside hydrolase families expressed by *T. virens* during maize interaction

Family	No. Proteins	Functions
3	4	beta-glucosidase, xylanase, several other functions
5	3	chitosanase, cellulase, xylanase, mannosidase, many other functions
11	3	endo-xylanase
12	3	endo-glucanase, xyloglucanase
27	4	galactosidase, isomalodextranase, arabinopyranosidase
31	3	glucosidase, sucrose-isomaltase, mannosidase, xylosidase
71	3	glucanase
79	3	glucuronidase, heparanase, several other functions
Other	35	chitinase, lysozyme, mannase, galactosidase, various other functions

The arsenal of cell wall degrading enzymes *T. virens* possesses is extensive and a large number of these are highly up-regulated during the interaction. The degradation products produced by this array of enzymes are linked to both signalling and nutrition. Signalling is mediated by the DAMP pathway and is activated in response to detection of cell wall polysaccharide degradation products by the plant (Boller & Felix, 2009). In our dataset maize produced xylanase and pectinase inhibitors, a direct response to cell wall degradation. Maize also appeared to up regulate both cellulose production enzymes and lignin deposition related enzymes. This may be an attempt by the plant to recover or adjust its cell wall to deal with the large scale degradation processes. Expansins and swollenin proteins were identified in the interaction, both of which are responsible for cell wall loosening, potentially facilitating enzymatic access to cell wall components or allowing cell penetration by the fungus (Cosgrove, 2000; Kang, Wang, Lai, Liu, & Xing, 2013). LRR proteins were also strongly up-regulated at 3 DPI, perhaps facilitating a signalling response to these enzymes. *T. virens* up-regulated several sugar transporters and metabolic proteins related to the breakdown of

polysaccharide components, suggesting that the fungus uses cell wall breakdown products for nutritional purposes. Sugar transporters may also play a role in uptake of polysaccharide signalling molecules such as sucrose and trehalose which appear to be able to regulate immune responses (Bolouri-Moghaddam, Le Roy, Xiang, Rolland, & Van den Ende, 2010). A number of proteins that facilitate but are not directly involved in cell wall degradation, such as acetyl xylan esterases, were also produced by the fungus.

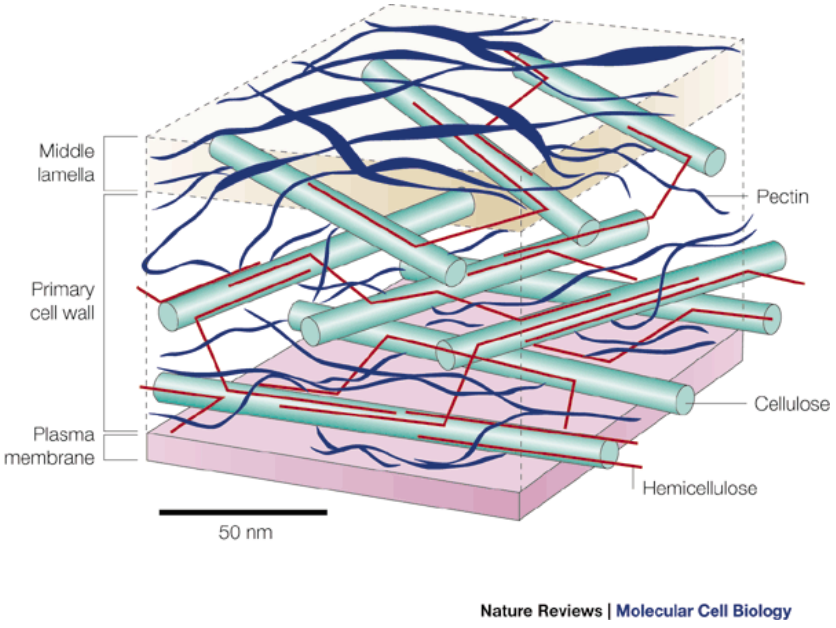


Figure 38: A diagram of a generic plant cell wall structure (Smith, 2001). Pectin, cellulose and hemicellulose are the main constituents of the plant cell wall. These are often protected by attached polysaccharide groups such as xylan. Lignin fills the spaces between these components and is also produced in response to cell wall damage in a Jasmonic acid and ROS mediated process (Denness et al., 2011).

A variety of secondary metabolites were produced by both *Trichoderma* and maize, suggesting a secondary metabolite communication pathway may have a strong influence on the interaction. Phenolic compounds were highly up-regulated in the maize plant during the interaction. These are responsible for direct toxicity against pathogens, but are also precursors to processes such as lignin deposition in the cell wall (Bhattacharya et al., 2010). Plant hormones were produced by both the plant and fungus; however it was difficult to demonstrate entire pathways being up-regulated. A subtle but intriguing link was the repression of gibberellins in maize paired with the production of related pathway products in *Trichoderma.* Due to the antagonism of these products to plant defence process, it is plausible that the fungus is attempting to reduce defence hormone signalling in the plant (**Figure 39**). The diverse range of other secondary metabolites related to defence, such

as kynurenines, coumarates, flavenoids and shikimate derivatives, provides compelling evidence for volatile based cross-communication, albeit in a manner that is hard to elucidate from this study.

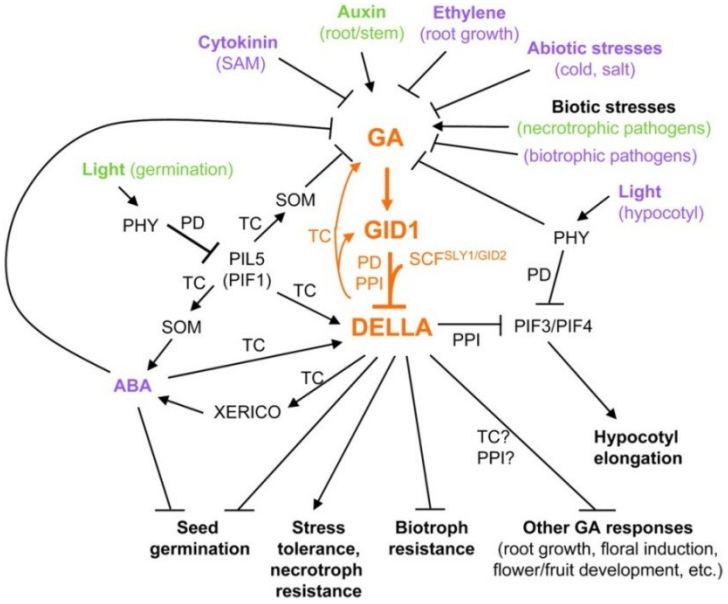


Figure 39: A diagrammatic explanation of gibberellin signalling pathways, mediated via the DELLA protein. Abbreviations are as follows – PD – Protein degradation, PPI – Protein-protein interaction, TC – transcription, SAM – Shoot apical meristem (Sun, 2010). This shows the intricate network of plant hormone regulatory processes. JA, GA, ethylene and SA were all transcriptionally regulated in the 3 DPI interaction. This implies hormonal responses to root colonisation although the exact pathway was not clearly demonstrated by these results. Image sourced from Tai-ping Sun (2010).

Perhaps the most surprising of the up-regulated pathways was the number of toxin and toxin resistance genes expressed by maize and *Trichoderma*. As endophytic interactions are symbiotic the production of a consistently and strongly up-regulated aflatoxin pathway suggests that toxins may be used to weaken hosts defences, particularly during initial colonisation. A number of related but equally potent toxins such as HC toxin (and potential biosynthetic precursors), tricothecenes and gliotoxins were present in the dataset, however of these only the tricothecenes were up-regulated. The effects of deletion of these toxins from *Trichoderma* upon plant interaction may prove interesting and possibly necessary if *T. virens* was to be used as a mainstream biocontrol agent.

The final consistently up-regulated group is also the most elusive. A group of proteins of unknown function were consistently up-regulated during the interaction. A number of these were small potentially secreted proteins, which may indicate they are effector proteins. **Figure 40** shows a possible mechanism of action for effector proteins. These would interact with the LRR proteins in the plant to block or repress immune functions. As several LRR proteins were repressed, these unknown proteins would provide good candidates for novel effector discovery.

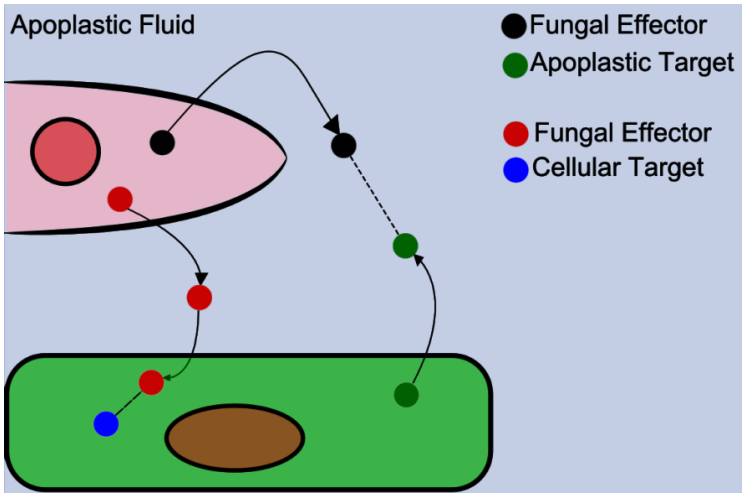


Figure 40: Effector translocation and targeting from fungal cell to plant host. Effector proteins are released by the fungus into the cytoplasm. From here they may directly interact with host apoplastic targets, i.e. by degrading host proteases such as serine proteases. They may also be translocated across the plant cell wall where they may interact to interfere with host machinery such as transcription factors.

5.6 Conclusion and Future Work

This study has demonstrated clear evidence for plant polysaccharide degradation as a predominant mechanism for fungal entry and nutrition during endophytism. Several pathways, particularly volatiles that may be crucial to communication were also identified. A firm basis for future molecular studies of highly up-regulated molecules identified in this study has been established. The multiple redundancies of the polysaccharide degrading compounds does however, make future molecular studies on them more technically difficult. Polysaccharide uptake transporters provide simpler targets and may also be selected to interfere with specific polysaccharide functional groups. Volatile compounds may be similarly interesting to examine, as removal of certain biosynthetic precursors should cause a number of metabolic and signalling changes in this system.

6 Molecular Characterisation of XlnR1-Like Proteins

6.1 Abstract

Transcriptome analysis was used to identify genes that were up-regulated by *T. virens* during root colonisation. Two candidate genes, TV_47927 and TV_58714 were selected from this data, to attempt to characterise their function in the interaction. Both genes encoded xylanase regulators that were similar to the Xyr1 regulator of *T. reesei.* These were selected due to the strong transcriptional evidence suggesting that cell wall degradation had a primary role in endophytic colonisation of maize. As a large number of CWDEs were expressed and likely to provide functional redundancy, deletion of the regulators was hypothesized to be more likely to cause a detectable phenotype in deletion mutants. Attempts to transform *T. virens* Gv 29.8 were hampered initially by poor protoplasting results, and a procedure was optimised for *T. virens* strain Gv29.8. Subsequently, transformation was unsuccessfully attempted using a vector-directed method. Direct transformation of PCR products proved more successful; however only recombinants containing ectopic mutations were obtained. The lack of homologous recombinants prevented functional characterisation from being completed.

6.2 Introduction

Two genes, TV_47927 and TV_58714 were selected from genes that were up-regulated in **Chapter 5**. Both targeted genes were from a known group of xylanase regulators with homology to Xyr1 of *T. reesei*, which is itself an orthologue of the XlnR1 regulator of *Aspergillus niger* (Stricker, Grosstessner-Hain, Wurleitner, & Mach, 2006). Regulators of this type have been well studied for their role in industrial cellulase production, but not commonly in their role in endophytic colonisation of plants (Seiboth & Seidl-Seiboth, 2011). CWDEs were implicated as significant factors in the establishment of root colonisation, as they were up-regulated across all measured time points. Due to the large redundancy of CWDEs (77 expressed at 3 DPI alone) the xylanase regulators were considered to be a more effective target for deletion. Multiple redundancies of CWDEs suggested that deletion of any one CWDE encoding gene would have little effect, whereas deregulation of the CWDE process was considered to be more likely to cause a visible phenotype (Carapito et al., 2008).

Regulators of the Xyr1 and Xlnr1 type usually contain a GAL4 zinc cluster domain and a fungal specific transcription factor domain (van Peij, Gielkens, de Vries, Visser, & de Graaff, 1998). GAL4 is most widely studied in yeast, and functions as part of a repressor-based regulatory system. In the absence of galactose a repressor binds to the GAL4 region preventing transcriptional activation. The repressor is removed upon exposure to galactose via an as yet unknown mechanism (Traven, Jelicic, & Sopta, 2006). A similar mechanism has been presented in *T. reesei* Xyr1, where a frameshift mutation in the GAL4 region disabled regulatory activation of the cellulase pathway (Lichius et al., 2015). The action of these regulators has been studied in cellulytic systems. Xyr1 acts in conjunction with ace2 to activate transcription of cellulases in response to the detection of cell wall polysaccharides such as galactose (Stricker et al., 2006). Loss of function mutations of XlnR1 in *A. niger* showed severely reduced xylanase transcription (van Peij et al., 1998). XlnR1 regulators have been isolated in *Aspergillus niger* and *Aspergillus tubingensis*, where they were shown to have a strong influence on the ability of these fungi to grow on and metabolise xylan related molecules (de Souza et al., 2013; Peij et al., n.d.; Van Peij, Visser, & De Graaff, 1998). A deletion mutant of a similar regulator, Xyr1 in *T. reesei* was shown to have significantly reduced capacity to metabolise D-xylose and xylan, which are both key plant cell wall components (Stricker et al., 2006, p. 1). Xyloglucan, a polymer formed of D-xylose and β-glucans, is the most common cell wall component of all known plants, forming the majority of the hemicellulose layer (Ochoa-Villarreal et al., 2012). Xylanase regulators are therefore likely to have highly relevant effects upon root colonisation by regulation of the large number of lytic enzymes detected via transcriptome analysis. Although their influence on fungal metabolism has been widely reported, the effect of deregulation of the xylanase system on plant colonisation ability has not been examined.

Genetic transformation of fungi has become a common method of studying fungal molecular biology. Most techniques used for this originated in yeast-based systems, particularly those designed to transform *Saccharomyces cerevisiae*, but several other fungi (*Aspergillus*, *Neurospora* and *Trichoderma* species amongst others) have been successfully transformed (Fincham, 1989; Ruiz-Díez, 2002). The most commonly used techniques are protoplasting, electroporation and biolistics, which have all be trialled on a variety of species with varying degrees of success (Ruiz-Díez, 2002). Protoplast transformation, has previously been successfully used to transform and create several deletion mutants of *Trichoderma virens* (strains Gv 29.8 and 10.4) (Baek & Kenerley, 1998; Djonović, Pozo, Dangott, et al., 2006; Djonović, Pozo, & Kenerley, 2006; Mendoza-Mendoza et al., 2003). Considerable species and strain based variation exists in the success rate of each

technique, with the success of protoplast transformation varying based on the quality and number of protoplasts. The quality and number of protoplasts is in turn dependant on the enzyme used, osmotic media and the fungus' propensity to take up foreign DNA (Fincham, 1989). Protoplast transformation was suggested as a preferred method for *Trichoderma* spp. (Artemio Mendoza, personal communication).

Two gene deletions were attempted in this study (TV_47927 and TV_58714), using PEG mediated transformation of *T. virens* Gv29.8 protoplasts. It was hypothesized that the deletion of two xylanase regulators should disrupt CWDE production in *T. virens*, potentially limiting cell penetration.

6.3 Methods

6.3.1 Identification and Comparison of Candidate Genes

Regulators of CWDE activity were identified by manual search, and two candidates homologous to Xyr1 and related to XlnR1 were identified. These candidate genes were TV_47927 and TV_58714. TV_58714 is up-regulated throughout the interaction, whereas TV_47927 is up-regulated from 5 DPI onward.

The two genes were aligned with Xyr1 in MEGA6 (using the default settings; gap opening penalty 15, gap extension penalty 6.66, IUB DNA weight matrix) to examine differences in the protein structure. Phylogenetic trees were constructed to compare these proteins in *Trichoderma* and other fungal groups. PRANK was used to generate alignments for phylogenies and BEAST was used to create maximum likelihood trees (Bootstrap value of 100).

6.3.2 Gene Characterisation Strategy

Once genes were selected, a strategy for characterisation was determined. It was decided to attempt to create deletion mutants of the TV_47927 and TV_58714 genes in *T. virens* Gv29.8, in order to determine if these two genes were required for root colonisation.

6.3.3 Construction of Inserts for Transformation

To generate deletion constructs, initial inserts were created by amplification of 1100 kb sequences flanking each gene (TV_47927 and TV_58714), using genomic DNA obtained from *T.virens* via the method in **section 2.6.1**. Amplification was performed using primer pairs 47927 LF-L + 47927 LFR – SfiI, 47927 RF-L SfiI + 47927 RFR for TV_47927 and 58714 LF-L + 58714 LFR – SfiI, 58714 RF-L SfiI +

58714 RFR for TV_47927 (**Table 6**). Flanking regions were then ligated to a hygromycin B resistance cassette using the protocol shown in **Table 8**. Unfortunately, ligation (**Figure 41 C**) yielded few ligation products of the appropriate size (4.7 kb) and attempts to re-amplify the full length insert using the initial PCR products as a template were unsuccessful. An alternative strategy was pursued (**Figure 41 D**) using overlapping PCR of two initial PCR products, each containing overlapping sections of the hygromycin B cassette. Overlap PCR products (2.8 kb) were then ligated (**Table 8**) into the vectors described in **section 2.5**. and verified via DNA sequencing using M13 universal primers either side of the insert.

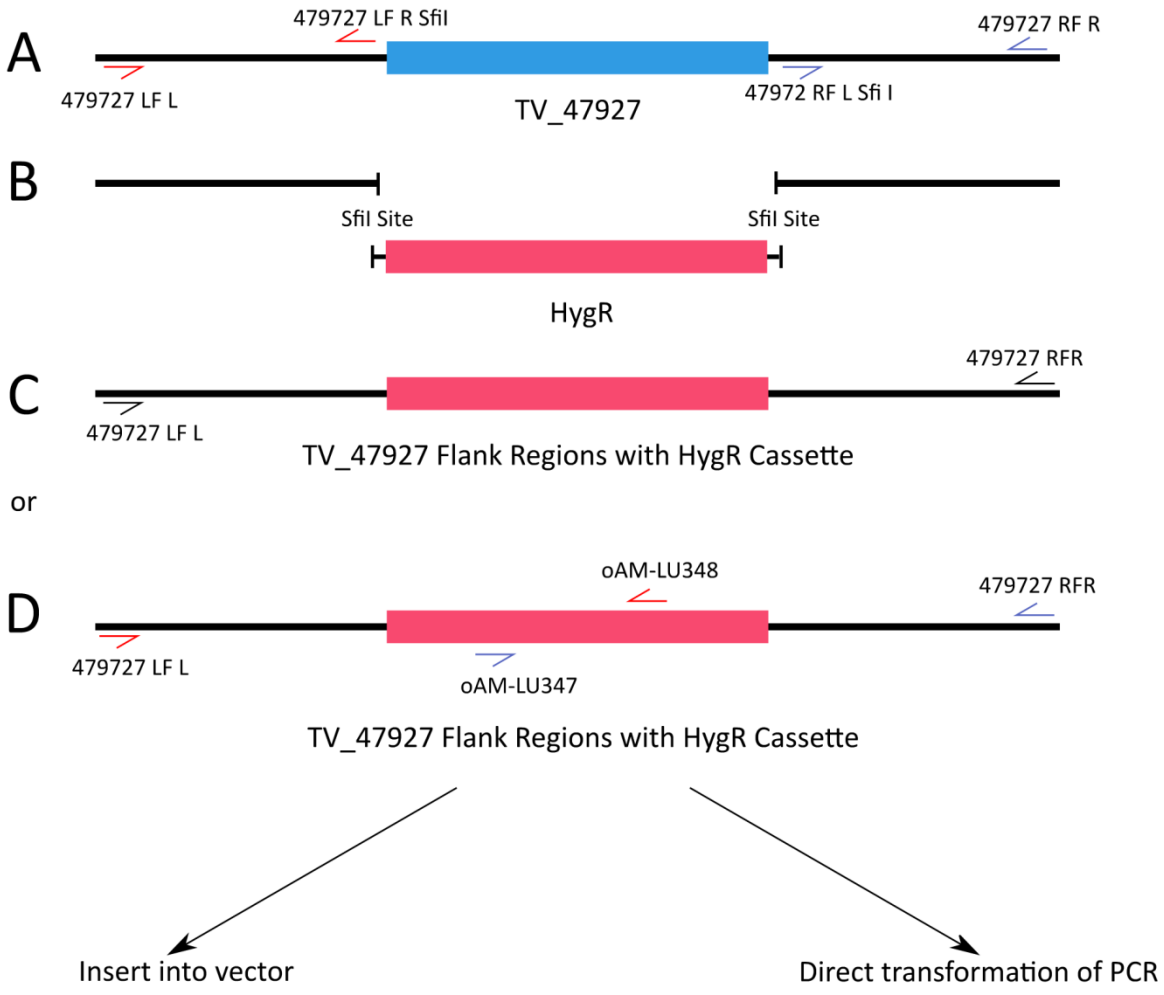


Figure 41: Workflow for creation of constructs for disruption of the Xyr regulators. The target gene plus 1100bp 3' and 5'were amplified using PCR (**A**). Primers were engineered to contain SfiI restriction sites which were used to cut out the gene of interest and replace it with a hygromycin cassette containing matching sites (**B**). The first strategy (**C**) involved an insert of approximately 4500bp in one construct. Strategy **D** involved creating two overlapping inserts, each of which contained a full length flank region and half of the resistance cassette. Small arrows indicate primer pairs used.

6.3.4 Protoplast Isolation

6.3.4.1 Initial Protoplast Isolation Method (Method One)

Trichoderma virens Gv 29.8 spores were inoculated onto PDA and grown for seven days. Spores were harvested by suspension in 10 mL of sterile water followed by passage through two layers of sterile miracloth. Glucose-Yeast Extract-Casein (GYEC) media (**Table 3**) was sterilised by autoclaving. Following this 100 mL of media was poured into sterile 200 mL flasks and inoculated with 1 mL of *T. virens* spore suspension. Flasks were incubated 15 h at 25^{o}C and shaking at 180 RPM. Mycelia were then harvested by filtration through two layers of sterile miracloth. Mycelia were then washed with 50 mL H_2O followed by a second wash with 50 mL of 0.01 M phosphate buffer at pH 7.2. Mycelia were then removed from the filter paper and 0.6g of mycelia was then suspended in an enzymatic solution of 36 mL mannitol osmoticum (0.5 M) (**section 2.3.1**) and 360 mg Glucanex. This was incubated for 4 h at 25^{o}C and shaken at 200 RPM. Protoplasts were then recovered by filtration through miracloth followed by centrifugation at 8000 RPM.

6.3.4.2 Final Protoplast Isolation Method (Method Two)

Mycelial material was prepared by inoculation of 100 µL of spores onto the centre of a large (150mm x 150mm) PDA plate covered with sterile cellophane and then spread using a triangular spreader. Plates were left to grow for 15 h before the cellophane was removed. Digestion solution was prepared; it consisted of 0.24g cellulase and 0.5g Glucanex (Novozyme) in 50 mL 0.7 M mannitol osmoticum. The cellophane was immersed in 10 mL of digestion solution from this 50 mL stock. The digestion mix was incubated for 4 h at 25^{o}C shaking at 150 RPM. Protoplasts were then recovered by filtration through miracloth, followed by a second filtration through a Swinnex filter using 40 µm nylon mesh. The protoplast suspension was then centrifuged at 4000 RPM for 10 min. A pellet formed, and the supernatant was decanted. The pellet was then suspended in 500 µL of 0.7 M mannitol osmoticum (**section 2.3.1**). Protoplast yield was then counted using a haemocytometer, counting five of the ten squares and averaging the result. Protoplasts were then stored by freezing in ½ volume polyethylene glycol (PEG) 4000 and 15 µL DMSO solution.

6.3.5 Optimisation of Protoplasting Methodology

Protoplast formation using the initial method was yielding insufficient numbers of protoplasts for transformation. Procedures were developed to optimize the protoplast procedure. The final **method two** was determined by the results of the experiments below. **Section 6.3.5.1** led to the

replacement of GYEC with PDA coated with cellophane as the growth media. **Section 6.3.5.2** led to the use of 0.7 M mannitol osmoticum as the buffer (replacing OM media). To investigate why the method had been less successful in *T. virens* Gv29.8 a cross-species and strain comparison was carried out in **section 6.3.5.3**. **Method 2** has become the standard protoplasting protocol in our lab.

6.3.5.1 Effects of Growth Media and Digestion Period on Protoplast Formation

Protoplasts were grown on PDB, GYEC and PDA (**section 2.3.2**) coated with cellophane to compare their growth characteristics and suitability for protoplasting. PDB was trialled with both steel wool and steel coils as these have previously been used to reduce the formation of aggregates in bacteria (Andrew Pitman, personal communication). Mycelia were grown on cellophane for one and two day time periods to determine the optimum time for this method.

6.3.5.2 Comparison of Buffer Effects on Protoplasts

Three osmotic media (OM Buffer, 0.5 M Mannitol Osmoticum and 0.7 M Mannitol Osmoticum (**section 2.3.1**)) were prepared. Protoplasts were then grown according to both **methods one** and **two,** substituting the osmotic media for those stated above. Yields were counted via haemocytometer, counting five of the ten squares and averaging the result.

6.3.5.3 Comparison of Successful Protoplast Formation for Six Trichoderma Strains from Four Species

Spores of six isolates of *Trichoderma* (**Table 2**) were plated on small (40mm x 40mm) circular plates of PDA and grown for four days in an incubator, with a 12 h day/night cycle at 25^{o}C. Spores were harvested by suspension in 10 mL H_2O followed by filtration through two layers of sterile Miracloth. Strains were then protoplasted by both **Method one** and **two** to determine species and strain specific differences in protoplast formation.

6.3.6 Transformation of *T. virens*

Ten ng of insert DNA was added to 240 µL of protoplasts (10^8 protoplasts) in 0.7 M mannitol osmoticum. Plasmid containing the insert was linearised via 1 h NotI digestion at 37^{o}C (40 µLplasmid, 6 µL bovine serum albumin, 6 µL NEB buffer 3, 1 µL NotI and 7 µL H_2O) and then cleaned using Wizard SV Gel PCR cleanup kit before use. However, due to low numbers of transformants this method generated, it was substituted by direct transformation using PCR product. The insert DNA + protoplast solution was then incubated on ice for 20 min, following which 130 µL of PEG

3500 was added and mixed very gently by inversion. A further 130 µL of PEG was added and the tube left to incubate for 30 min at room temperature. The transformation solution was then plated by mixture into 10 mL of overlay solution consisting of PDA + 200 µg/mL hygromycin. The mixture was then poured onto prepared plates containing 10 mL PDA + 200 µg/mL hygromycin. Hygromycin concentration was later dropped to 100 µg/mL to determine if the strong selection pressure at a concentration of 200 µg/mL was selecting for ectopic integration. A positive control plate consisting of PDA and protoplasts was used, as well as a negative control of protoplasts on PDA + hygromycin. Plates were grown for seven days at 25^{o}C before single colony selection was performed.

6.3.7 Selection and Confirmation of Transformants

Colonies were then selected and purified seven times by alternately plating on PDA + hygromycin and PDA, selecting single colonies on PDA + hygromycin and selecting single spores on PDA. This was to ensure a stable single transformant colony was isolated.

DNA was extracted from successful transformants using standard phenol:chloroform techniques (**section 2.6.1**). Transformants were then verified using PCR to determine whether the gene was deleted and if any non-target copies had inserted into the genome. Three PCRs were performed to verify the insert (**Figure 42)**. The first PCR amplified the across the insert using primers 47927 LFL and RFR (or 58714 LFL and RFR), indicating successful transformation. The second PCR amplified from outside the insert on the flank regions of the wildtype gene (primer pairs 47927 OPL and OPR or 58714 OPL and OPR). When digested with SfiI this should yield a 1.4 kb band for the hygromycin resistance cassette and a 1.7 kb band for the flank regions. The third PCR amplified outside the insert, to the hygromycin cassette (primer pairs 47927 OPL and oAM-LU347 or 59714 OPL and oAM-LU347). This allows verification of transformations and homologous integrations. Had a successful insert been detected a southern blot would have been used to further confirm it. An exterior amplification was also performed to determine the insert was correct. The resulting product was then digested by SfiI restriction enzyme to determine the correct insert was present. The original gene was then amplified using internal primers to determine if it had been replaced.

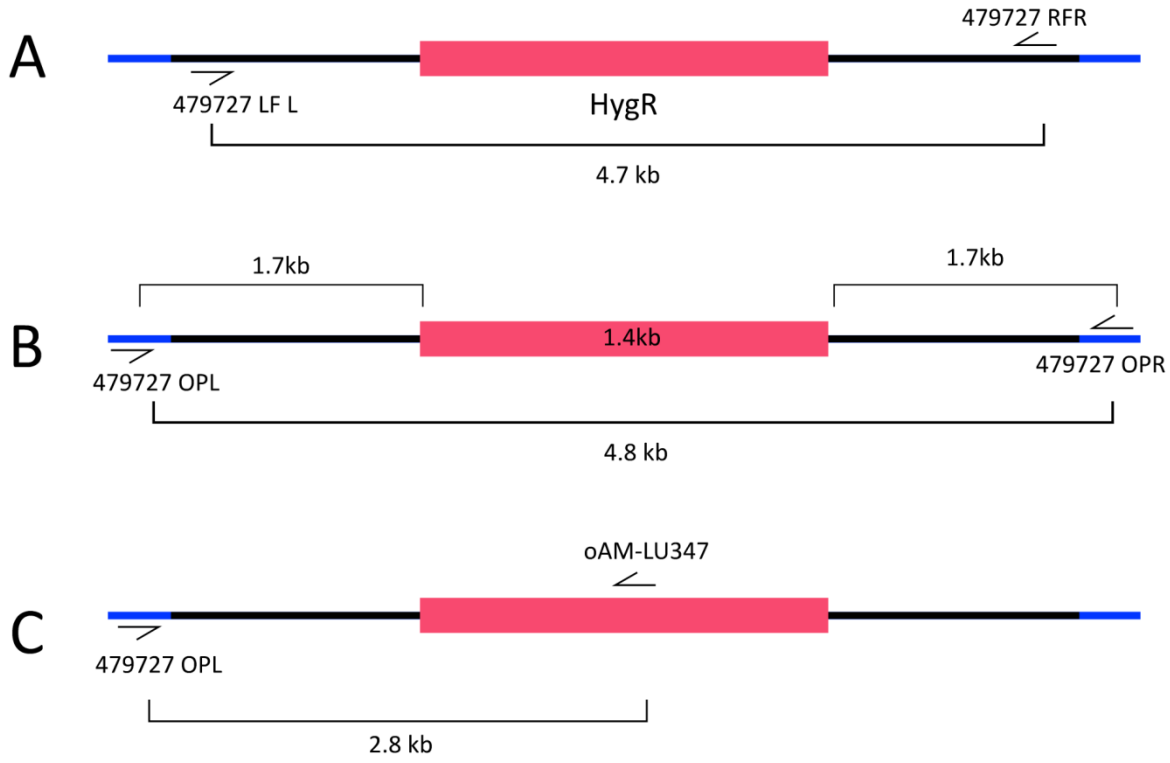


Figure 42: **Diagram showing primer locations for gene verification**. PCR **A** amplifies within the insert, determining if transformation was successful. PCR **B** amplifies in flank regions external to the insert, and when digested with SfiI confirms if integration was homologous. PCR **C** supports the finding of **B** by amplifying externally but also within the insert, confirming homologous insertion and the presence of an insert if homologous. Black lines represent regions inside the insert, blue lines represent external regions.

6.4 Results

6.4.1 Identification of Candidate Genes

Candidate genes were selected due to their ability to regulate a large class of lytic enzymes, which were identified as a major factor in the interaction between plant and fungus in the transcriptome data (see **Chapter 5**). Genes TV_47927 and TV_58714 both have sequence similarity to XyR1-and XlnR1 type xylanolytic regulators and are fungal specific transcription factors. TV_47927 encodes a 575 amino acid protein that has homology to a XlnR transcriptional activator class protein in *Trichoderma harzianum* (KKP01019.1, BLASTp, 97% identity). TV_58714 encodes a similar protein, of 922 amino acids, which has blast matches to several xylan regulators in *Trichoderma* spp. including *T. atroviride* (XP_013941705.1), *T. harzianum* (KKO98630.1) and *T. reesei* (TR_122208). The two proteins are differentiated by truncation at the start of TV_47927, with 321 amino acids in this region being absent (**Figure 43**).

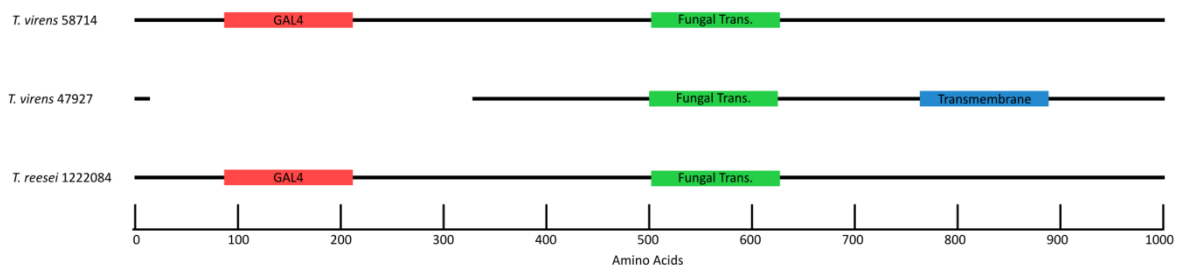


Figure 43: A sequence alignment representation of Xyr-like genes in *T. virens* compared to *T. reesei* *T. virens* 47927 is missing the GAL4 sequence of the other two genes, but has sequence similarity to both, and still contains the fungal transcription factor domain. It also has an extra transmembrane domain.

This region encodes a GAL4-like zinc-cysteine cluster that is common to many eukaryotic transcriptional regulators (Traven et al., 2006). In yeast this is responsible for the activation of galactose metabolism upon exposure to galactose. This mechanism would be logical in *Trichoderma*, where regulatory activation would occur upon exposure to the substrate, in this case the plant cell wall. The lack of the GAL4 region in TV_47927 may indicate that this gene either has an alternate form of regulation or is of different function. Both genes possess known fungal transcription factor domains. Phylogenetic trees were constructed from proteins with blast matches to the two candidate gene (see **Supplementary Material**). These showed clustering of each *T. virens* gene into two separate groups. *Trichoderma* Xyr genes cluster into one group with XlnR1 genes into a separate group including *T. atroviride* (XP_013941705.1), *T. harzianum* (KKO98630.1), *T. reesei* (XP_006966092.1), *T. koningii* (AFM31002.1) and *T. virens* (TV_58714). Closely related groups included *Fusarium* and *Nectria* species.

6.4.2 Protoplast Improvement

The initial method (**Method 1**) gave low protoplast yields which reduced transformation efficiency. Several factors potentially affecting the efficiency of protoplast formation in *T. virens* were identified, including growth media, osmotic stability and resistance to enzymatic digestion. Examination of *T. virens* in the standard GYEC growth media revealed that it formed aggregates in this solution, which were often large enough to be visible to the naked eye. Microscope images were taken of *T. virens* in GYEC, PDB (PDB, PDB + steel coils, PDB + steel wool), and PDA (**Figure 44**) to determine if aggregation could be prevented.

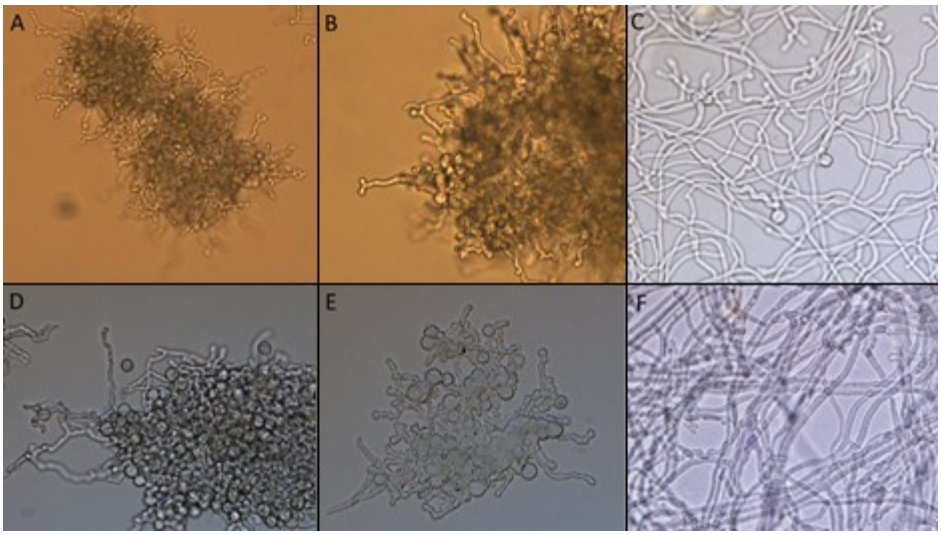


Figure 44: Microscopic visualisation of the effects of growth media on protoplast formation for *T. virens.* Microscope images of *T. virens* in various growth media at 40x magnification **A**) PDB, **B**) GYEC, **C**) Cellophane (1 Day), **D**) PDB + Steel Wool, **E**) PDB + Steel Coils and **F**) Cellophane (2 days). The key finding is the reduced aggregation of *T. virens* on solid media covered with cellophane. It was suspected that aggregates of *T. virens* are somewhat protected from enzymatic degradation, thus reducing aggregation should enhance protoplast formation.

Aggregation was limited by PDB solutions containing steel coils, but mitigated entirely by short periods of growth on cellophane covered PDA. Cellophane growth caused a larger surface area to be exposed to enzymatic degradation, increasing protoplast formation. Cellophane covered PDA was thus chosen as the major growth substrate for further protoplast experiments.

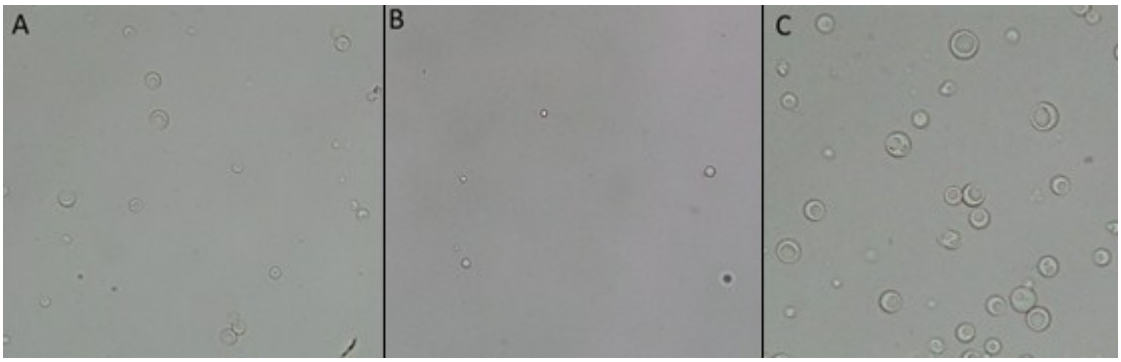


Figure 45: A comparison of osmotic media for use in protoplast formation. Microscope images of *T. virens* in different osmotic solutions at 40x magnification. **A**) Mannitol osmoticum at 0.5 M, **B**) OM media, **C**) Mannitol Osmoticum at 0.7 M. Mannitol osmoticum at 0.7 M was visibly resulting in more stable and numerous protoplasts and was used in subsequent experiments.

Osmotic media was the second variable that affected *T. virens* protoplast formation. Osmotic media affects the quality of protoplasts and their durability, as well as potentially affecting enzyme activity. OM media, comprised of a 0.6 M solution of mainly Magnesium ions, showed much less protoplast formation to visual observation than mannitol osmoticum at 0.5 M or 0.7 M (**Figure 45**).

OM media was used for initial transformation attempts, and may explain their failure, due to the low protoplast number and quality. Osmotic pressure appeared to be a key factor as 0.7 M mannitol was more effective than 0.5 M mannitol at stabilising protoplasts in the solution. Mannitol osmoticum at 0.7 M was therefore selected as the primary candidate for the final osmotic media, although it was noted that others have reported different results with other species of fungi as well as with other osmotic buffers (KCl for example may further increase protoplast recovery) (Lalithakumari, Mrinalini, Chandra, & Annamalai, 1996).

Protoplast recovery using the new method was higher than that of methods reported in several other papers. However, it was not as high as that reported for some fungal species (Lalithakumari et al., 1996; Natesan Balasubramanian, 2008). To determine if *T. virens* was relatively more difficult to protoplast compared to other species, an examination was made comprising six fungal strains from various *Trichoderma* species.

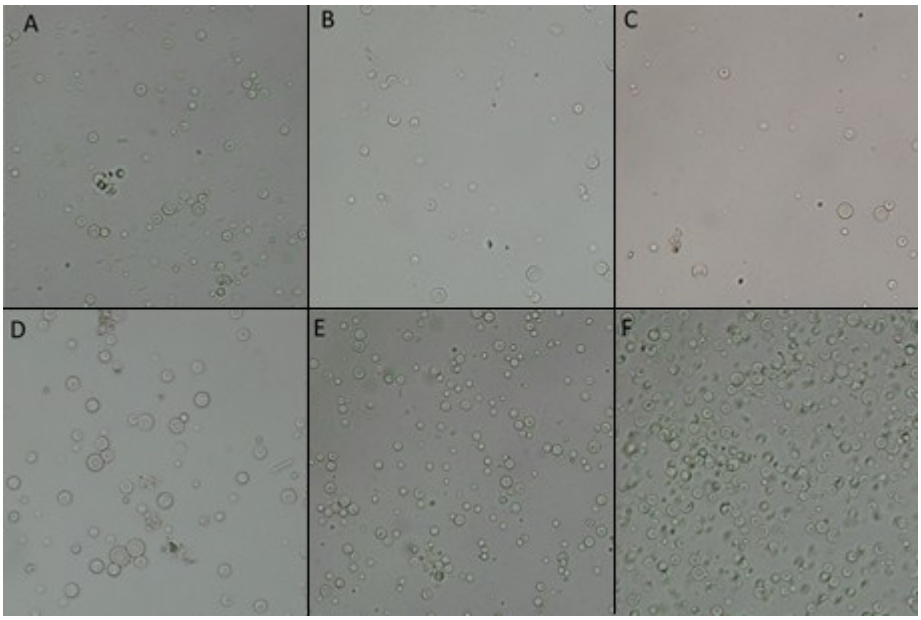


Figure 46: Microscope visualisation of protoplast formation in various *Trichoderma* Strains using the new protoplast method Microscope images of *T. virens* protoplasts generated using **Method 2** at 40x magnification. **A**) *T. virens* 10.4, **B**) T. virens 29.8, **C**) *T. hamatum* FCC207, **D**) *T. harzianum* FCC261, **E**) *T. atroviride* IMI206040 and **F**) *T. reesei* QM6. *T. reesei* QM6 had an order of magnitude higher protoplast counts than the other isolates. *T. hamatum* performed the worst with the lowest count at 2.5 x 106 protoplasts, followed by *T. virens* with 2.06 x 107 protoplasts. This provides some explanation for the difficulties in transforming *T. virens* via protoplast formation when compared to similar methods in IMI206040 and QM6.

The results of this protoplasting experiment are shown in both **Figure 46** and **Table 32**. *T. virens* is relatively resistant to enzymatic digestion compared to most strains tested. A protoplast count

average across three technical replicates is shown in **Table 32**. The two fungal strains with the lowest protoplast formation are *T. hamatum* and *T. virens*, with the former being two order of magnitudes lower than the most successful fungus, *T. reesei*.

Table 32: Protoplast counts in 6 *Trichoderma* strains following enzymatic digestion

Isolate	GYEC and OM Buffer Method (Protoplasts/mL)	Cellophane and Mannitol 0.7 M Method (Protoplasts/mL)	% Increase
FCC261	7.13×10^6	4.3×10^7	503
FCC207	1.0×10^6	2.5×10^6	150
Gv 29.8	4.57×10^6	2.06×10^7	350
Tv10.4	2.81×10^6	3.72×10^7	1223
QM6a	2.6×10^7	8.9×10^8	3323
IMI206040	2.1×10^6	3.75×10^7	1685

6.4.3 Fungal Transformation

Creation of *T. virens* transformants was successful, however due to the high ectopic mutation rate only non-homologous recombinants were obtained. Initial attempts to transform the fungus resulted in no successful transformants over several separate attempts. The quality and number of protoplasts formed seemed to be a major factor in this, which were remedied by the optimisation above. Once quality protoplasts were obtained the transformation proceeded reliably, generating approximately 20 hygromycin resistant colonies in each attempt (**Figure 47**). Unfortunately all appeared to contain ectopic mutations as well as the correct insert. No successful homologous transformants were confirmed at the time of writing.

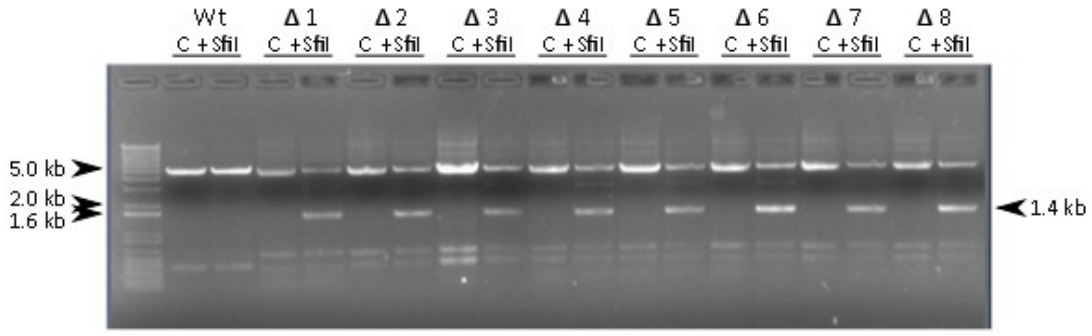


Figure 47: Gel of SfiI digested Hygromycin resistant mutant colonies. DNA was isolated from colonies resistant to hygromycin selection marker. PCR was run using primers 47972LFL and 47927LFR. Wild type (**wt**) and mutant (Δ) DNA was digested with SfiI. A control of undigested DNA was run alongside each sample (**C**) A 4.8kb band was seen in the wild type (lanes 1 and 2). A 1.4 kb band not seen in the SfiI digested wt was seen in the SfiI digested mutants. This indicated represented the hygromycin B cassette and indicated successful transformation.

Transformants were further analysed by PCR, this time amplifying across the exterior and digesting with SfiI according to the procedure in **Figure 42 B**. Lane four showed a single band (**Figure 48**), but it was not in the expected size range. Further confirmation was undertaken via a third PCR, from the exterior to the internal of the hygromycin cassette (**Figure 42, C)**. Amplification using this set of primers, resulted in no visualisable signal after gel electrophoresis. This suggests that the inset was inserted ectopically, resulting in hygromycin B resistance and the ability to undergo PCR amplification with primers 47927 LF L and 47927 RF R, but not deletion of the target gene. These results were typical of several attempts to obtain the correct insert and were observed by other members of the lab group attempting similar deletions (Guillermo Noguiera Lopez, personal communication).

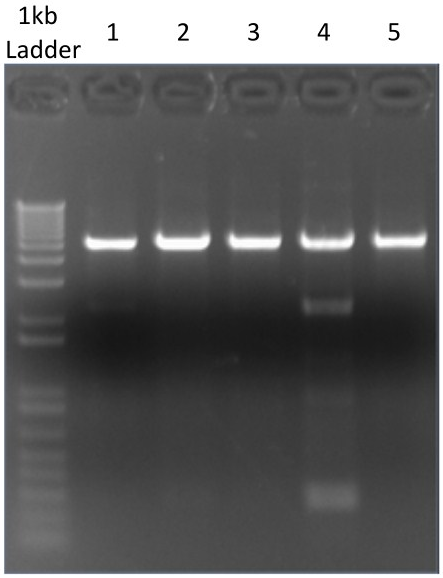


Figure 48: Gel of SfiI digested successful transformants. PCR using primers 47927 OPL and OPR resulted in no successful digestion, except for a fragment of the wrong size in lane four. This indicates mutants are ectopic, as they are hygromycin B resistant, but do not contain a homologous insert.

6.5 Discussion

Alignment of the TV_47927 and TV_58714 Xyr1 regulator-like genes showed two clear groupings amongst homologues of this protein in *Trichoderma* species, suggesting that these regulators may be of evolutionary significance to this genus. The ancestral importance of lytic enzymes to *Trichoderma* species (as fungi of saprobic origin) may be responsible for this grouping. However the identification of Xyr-like genes of *Fusarium* species and the discovery of several lytic enzymes involved in *Epichloe* colonisation of ryegrass, suggests that these regulators may be important to both pathogenic and mutualistic plant colonising fungi (Druzhinina et al., 2011; Kubicek et al., 2011). The truncation at the n-terminal end of the TV_47927 protein removed the GAL4 regulatory domain; however it retains sequence similarity to XlnR regulators, as well as the fungal transcription factor domain. This may suggest it forms a separate regulatory mechanism. However, without functional data it is impossible to say whether this protein is an alternative regulatory method, or simply a protein of different function.

Two xylanase regulator genes were selected for deletion, to avoid the problem of multiple redundancies in CWDEs that were differentially expressed in the *Trichoderma*-maize interaction. However, difficulties were encountered, firstly with protoplast generation, then with transformation. *Trichoderma* spp. have been successfully transformed using this method previously (Baek & Kenerley, 1998, p. 2; Mendoza-Mendoza et al., 2003, 2003; Wilhite, Lumsden, & Straney, 2001). Protoplasting was therefore optimised and three issues were identified. Firstly, the osmotic buffer used in method one was modified, as osmotic media has been a factor influencing the success of protoplast formation (Natesan Balasubramanian, 2008). Mannitol osmoticum at 0.7 M was determined to be the best of the osmotic media tested for this strain of *T. virens*. The second problem was caused by aggregation of mycelia in liquid media. Attempts to mitigate this using steel coils were ineffective when compared to simply growing mycelia for 24 h on PDA covered with cellophane. This was also advantageous in that it also decreased the growth period necessary for each protoplasting attempt and was more consistent. Protoplast formation was considerably more effective after the prevention of fungal aggregation via this method. It was then decided to examine the efficacy of this method across strains. *T. virens* Gv 29.8 was amongst the two strains most resistant to enzymatic degradation of the six strains tested. This may be linked to the mycoparasitic nature of *T. virens*, which uses cell wall digestion to attack prey species, thus requiring an ability to protect itself from these enzymes (Gruber & Seidl-Seiboth, 2012). This would be useful in

endophytic colonisation as it would confer resistance to plant proteases and masking processes related to this resistance may hide the fungus from plant sensors (de Jonge & Thomma, 2009). However, *T. reesei* is a saprobic fungus used industrially for the production of large quantities of cellulase and it had substantially higher protoplast counts, so broad spectrum enzymatic resistance may not explain the phenomenon. It is possible *T. virens* is simply more resistant to the enzyme mix used than other *Trichoderma* species. Regardless, the quality and number of protoplasts obtained was higher than that of some reported protoplast methods and yielded more protoplasts, with less cellular debris present in the mixture (Natesan Balasubramanian, 2008).

Unfortunately the primary goal of this study, the creation of a deletion mutant, was unsuccessful despite repeated attempts. Protoplasting problems were causing very low numbers of successful transformants to be isolated, which was solved by the use of the new method. However, further problems were encountered with high ectopic integration rates. Similar issues with ectopic mutations have been reported, however successful mutant were eventually obtained in other studies (Wilhite et al., 2001). Ectopic transformations were also a problem in other deletions attempted in the same lab (Guillermo Noguiera Lopez, personal communication). Alternate methods of transformation will be used to attempt to circumvent this issue, including gene-gun transformation and agrobacterium mediated transformation of protoplasts. Gene-gun transformation is reportedly effective at limiting ectopic mutations (Lorito, Hayes, Di Pietro, & Harman, 1993). The development of an Arg2 mutant was also initiated to attempt to enhance transformation success this issue (Baek & Kenerley, 1998). The inability to create a successful deletion mutant severely hampered the goal of this chapter, to characterise the effect of the two xylanase regulator genes on root colonisation.

6.6 Conclusion

Characterisation of Xyr genes by disruption was unsuccessful due to the prevalence of ectopic mutations. Because of this we were unable to confirm the function of the regulators selected from transcriptome data. Attempts to mitigate issues with protoplasting resulted in an enhanced protoplasting method for *T. virens* Gv 29.8 and identification of different rates of protoplast formation in different *Trichoderma* species. Problems with ectopic mutations were not overcome, but other methods of transformation such as biolistics are being investigated. Future work on these deletions will use alternate transformation methods to overcome the high ectopic mutation rate observed in these experiments.

7 Final Discussion

7.1 *Trichoderma virens* is an Endophyte of Maize for up to 7 DPI and Colonisation is Affected by Host Specific Factors

The first and most important aim of this study was to confirm that *T. virens* was capable of establishing an endophytic relationship with maize. *T. virens* was observed through fluorescence, confocal and electron microscopy, to be capable of growing both inside and on the surface of maize roots and shoots for periods of up to seven days. Surface colonisation was prevalent, with considerably less endophytic growth being observed. The extent of surface colonisation suggests that *T. virens* is more likely to spread throughout the plant by growth along the surface, rather than by endophytic growth. The fungus did not preferentially localise to either shoots or stems, however this may be an artefact of the inoculation of *T. virens* spores on seeds, as the predominantly colonised area was the 2 cm either side of the seed. In natural conditions it would seem likely that *T. virens* populations would be in contact with large areas of the root surface, and would thus be able to penetrate areas across the root surface.

At 7 DPI, plating assays identified the presence of *T. virens* up to 14 cm from the inoculation site in roots and 12 cm from the inoculation site in shoots. Therefore it appears likely that *T. virens* would be capable of colonising more distant regions of the plant, such as the upper stem, if sufficient time was allowed. This could be investigated by repeating the growth study for durations longer than seven days. The major issue with investigating this in maize is the rapid growth of maize plants in controlled conditions, which causes them to rapidly outgrow their containment chamber. *Trichoderma* species have been observed persisting in maize for up to 6 weeks (Sobowale A. A., 2011). In the author's opinion, this suggests that a temporal definition of endophytism should be applied, to differentiate between transient endophytes and those that persist for long periods, such as obligate endophytes.

Finally, the endophytic colonisation ability of *T. virens* appears to be host specific, and has an effect even between lines of the same plant species. *T. virens* was significantly more able to colonise four of the 15 maize lines tested (**p** = 0.01). This correlates well with other observations of *T. virens* line specificity in tomato and maize plants (Morán-Diez et al., 2015; Richard S Quilliam, 2012), as well as the host specificity observed in other endophytes (T. Zhang & Yao, 2015). Specific matching of

endophytic isolates to host lines may therefore be important in maximising the agricultural effect of mutualistic endophytic species. This is particularly relevant given the increased use of biocontrol agents in the past ten years (Chandler et al., 2011). It also suggests that endophytic species for use in an agricultural biocontrol role should be tested against a range of non-target species, as it has not been determined if mutualistic endophytism is a stable trait, or rather the result of each independent plant-fungal interaction (Delaye, García-Guzmán, & Heil, 2013).

7.2 Proposed Model of *T. virens* Host Entry

Trichoderma virens appears to fit the description of *Trichoderma* spp. as 'opportunistic avirulent endophytes' very well. *T. virens* is capable of colonising root surfaces extensively, with limited but frequent growth in the outer cell layers of the plant host. *Trichoderma virens* can enter the plant via multiple methods, which may all be operating at the same time (**Figure 49**); however growth is limited to the cortical tissue of maize. Digestion of the cell wall to facilitate entry allows access to intercellular spaces and provides a source of nutrition (**A** and **B**)**.** Transporter proteins may be expressed here to absorb breakdown products of the cell wall and may also be part of signalling pathways (Wahl *et al*., 2010). Formation of appressoria-like structures allows direct entry to the plant cell cytoplasm, where invasive hyphae-like structures form (**C**). These may allow nutrient acquisition and delivery of effector proteins into the plant cell that modulate the immune system (Catanzariti et al., 2007; Staples, 2001). Growth in plant inter-cellular spaces may be facilitated by the release of fungal effector proteins such as swollenins (**D**), which loosen plant cell walls (Brotman et al., 2008). Effectors such as LysM proteins and protease inhibitors may also be released here to protect the fungus from plant defences (de Jonge & Thomma, 2009). *Trichoderma* growth is limited to the cortical layers by the effective application of plant defences, such as lignification of cell walls (**E**) (Bhuiyan et al., 2009). Finally, extensive growth of the fungus along the plant surface allows rapid spread of the fungi along both root and stem tissue (**F**).

As previously discussed, growth on the plant surface (**Figure 49**, **F**) was the most prevalent form of *T. virens* growth observed by microscopy. Colonisation of the internal area of the host was observed in both intra- and inter-cellular spaces. Inter-cellular growth is a mechanism of fungal proliferation inside the plant host, and is supported by direct microscopic visualisation and by the up-regulation of expansins and CWDEs in the transcriptome data. CWDEs provide a mechanism for fungal entry at any point on the host plants surface, without the requirement for exploitation of weak points, such as stomata (Arnaud & Hwang, 2015; Gudesblat, Torres, & Vojnov, 2009). Once inside, expansins,

such as swollenin, are produced by other fungi to loosen plant cell walls (Brotman et al., 2008; Cosgrove, 2000). Inter-cellular growth was the second most prevalent form of growth in the interaction, and was predominantly in outer layers of the plant cortical region. No evidence of growth in vascular regions was observed, and this may be the result of plant responses such as cell wall lignification (**Figure 49**, **E**). Limited evidence for lignification and plant wall modification was obtained in the transcriptome study. The restriction of *T. virens* from vascular tissue is also unsurprising as it is a hallmark of either obligate endophytes or particularly damaging pathogenic species, and the lack of *T. virens* growth in this region may be a result of a successful plant immune responses (Yadeta & J. Thomma, 2013). Lignification and callose deposition are relatively common plant responses to invading microbes (Bhuiyan et al., 2009; Malinovsky et al., 2014). Further investigation of lignification and callose deposition would determine if these were indeed the factors limiting *T. virens* growth, and techniques for visualising both exist (Ellinger et al., 2013; Tobimatsu et al., 2013).

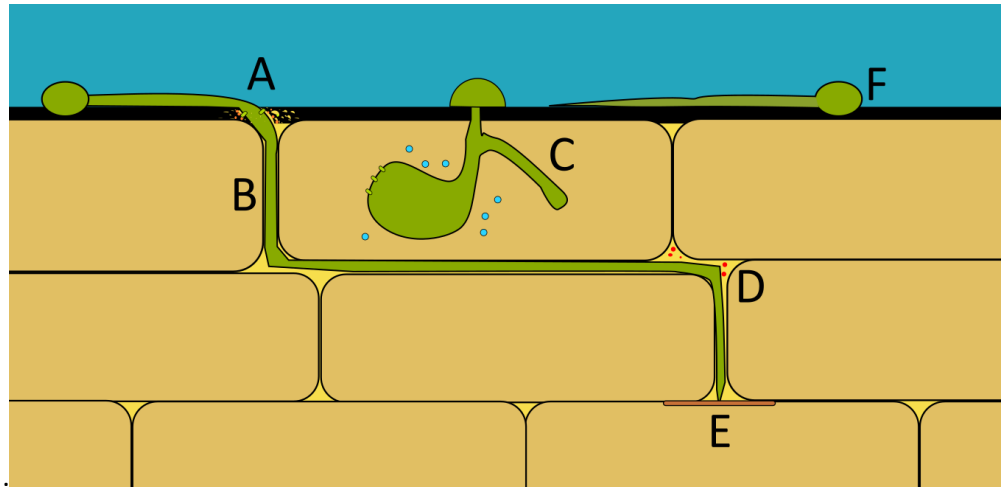


Figure 49: Methods of plant penetration used by *T. virens* Cell wall digesting enzymes allow penetration of the host (**A**) and access to intercellular spaces (**B**). Appressoria formation and invasive structure development allow penetration of intra-cellular spaces, nutrient acquisition and effector release (**C**). Intercellular growth is aided by swollenins and protective effectors (**D**), but is eventually restricted by plant lignification (**E**). *Trichoderma* can spread along the exterior of the plant, allowing rapid growth along root and shoot surfaces (**F**).

The final mechanism for host entry is appressoria formation (**Figure 49**, **C**). Structures that resembled appressoria and haustoria were visualised in *T. virens* by confocal microscopy. Only one clear appressoria-like structure was observed, making it unlikely that this is a primary method of plant penetration. Appressoria formation was not demonstrated at every site of penetration by *T. virens*, although appressoria may be transient structure and thus only present for short periods of

time. However, due to the proliferation of CWDEs shown by transcriptomics, it appears that cell wall degradation is a more likely penetration mechanism, which is also able to support penetration by appressoria (Hématy et al., 2009). Effectors and lytic enzymes may also be released by invasive hypae-like structures, and this is common in pathogens (Catanzariti et al., 2007). *Trichoderma* spp. are known to be capable of forming appressoria during mycoparasitism and the literature suggests that *Trichoderma* spp. also use these appressoria to penetrate their hosts in a manner similar to that of plant pathogens (Harman et al., 2004a). The visualisation of appressoria-like structures by *T. virens* during plant penetration does support the general assertion that endophytes employ similar host-entry mechanisms to plant pathogens (Lanver et al., 2010). Confirmation of the frequency of appressoria formation may be important in determining the significance of appressoria function to root colonisation. Visualisation of appressoria formation has been performed in *Magnaporthe* using actin binding fluorescent proteins (Dagdas et al., 2012), and may provide a method of increasing the detection frequency for similar structures in *Trichoderma*.

7.3 Proposed Mechanism of Molecular Communication Between *Trichoderma virens* and *Zea mays*

Trichoderma virens-maize communication appears to operate through several pathways (**Figure 50**). Polysaccharide signalling, plant hormones, MAPK and traditional defence pathways (NBS-LRR proteins) were all differentially expressed in maize during the interaction, while putative effectors, lytic enzymes, secondary metabolites and redox proteins were up-regulated in *T. virens*.

The evidence supporting CWDE and related pathways is considerable, suggesting that cell wall degradation plays a larger role in endophytic interactions than previously thought. *In silico* analysis predicted a group of CWDEs as secreted proteins, although these were removed from effector searches as they have known enzymatic function. Transcriptome analysis provided substantial evidence for CWDE up-regulation, with consistent up-regulation of approximately 33% of the glycoside hydrolase enzymes encoded in the *T. virens* genome. Furthermore, the range of such enzymes produced was capable of degrading a majority of known plant wall polysaccharides. Sugar transporters were also up-regulated, providing evidence for a nutritional role for cell wall degradation, and this may suggest polysaccharide signals play an important role in the interaction. Polysaccharide transport may also be a means by which the fungus attempts to mask the results of cell wall degradation from the plant, thus limiting the extent of the DAMP response.

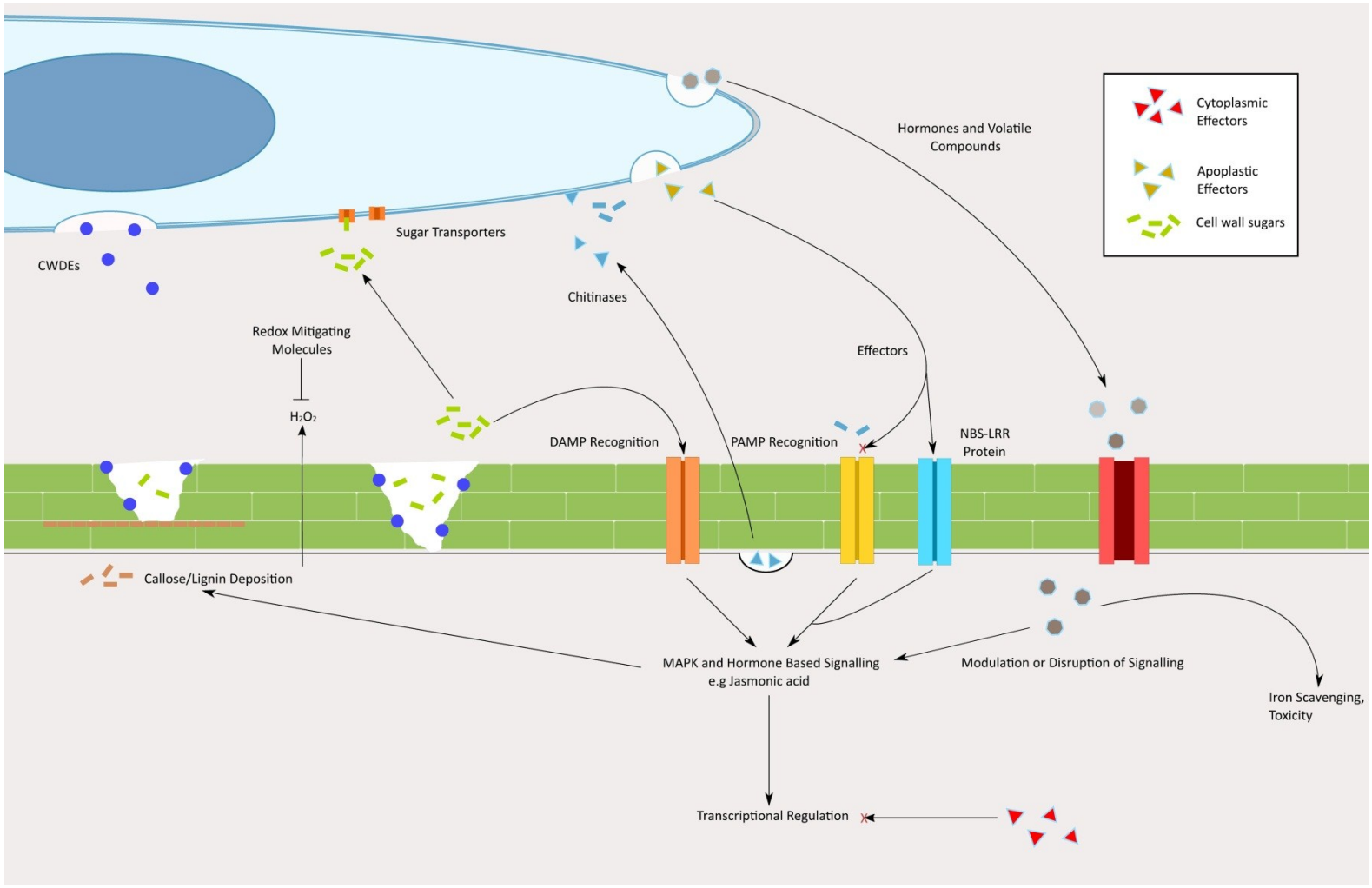


Figure 50: A mechanism for molecular communication between *T. virens* and *Z. mays*

Microscope evidence indirectly supports CWDEs as a major mechanism, as the extent of *T. virens* penetration was not explained by other physical mechanisms. The up-regulation of CWDEs in endophytes has been recently observed in *Epichloe* (Eaton et al., 2014), however the production of CWDEs is more commonly associated with pathogenic fungi (Hématy et al., 2009; Malinovsky et al., 2014). Cell wall damage is responsible for triggering of DAMP signalling pathways and, when combined with transporters, for polysaccharide signalling which may implicate CWDEs as a method for communication between fungus and host (Thonart et al., 2012; Tognetti, Pontis, & Martínez-Noël, 2013; Wahl et al., 2010). Preliminary experiments were performed to test the effects of sucrose on the interaction (data not shown), and extending these may be a way to confirm the role of polysaccharide signalling.

Further support for the role of CWDE in signalling comes from the plant transcriptome. Limited evidence was obtained for lignification of plant cell walls, as well as the up-regulation of metabolic pathways that may be related to cell wall modification. Lignification or modification of plant cells may restrict *Trichoderma* from penetrating beyond cortical layers. Cell wall modification (such as lignification) can be initiated by DAMP recognition. DAMP recognition provides a mechanism for a plant to detect an organism through the damage it causes to the plant, rather than by detection of the organism itself (Heil & Land, 2014). Detection occurs by the recognition of small polysaccharide molecules or pectins, which then elicit an immune response (Hann, Bequette, Dombrowski, & Stratmann, 2014). Various signalling enzymes, such as MAPK, which often have signalling roles in response to such pathways, were also up-regulated, but could not be clearly linked to DAMP pathways using the methods in this study. Hormones such as jasmonic acid, were also differentially expressed, but not clearly linked to CWDEs by this study. Jasmonic acid in particular is known to respond to DAMP recognition (Koo & Howe, 2009).

Large numbers of genes encoding proteins of unknown function were differentially expressed during the interaction. These genes were considered interesting predominantly for the likelihood that some had a role as effector molecules. Bioinformatics identified proteins of unknown function as the third largest group of proteins in the *T. virens* genome. Despite the large numbers identified, the difficulty of using predictive measures on proteins with no function became apparent. Effectors were predicted by small size (300 amino acids), no function and the presence of a secretion signal. However, when compared to transcriptome data, only 180 of the 726 predicted proteins were found in both datasets. Comparison of bioinformatics data to transcriptome data yielded a

relatively poor discovery rate of 24% for genes encoding unknown proteins involved in the interaction, and the percentage that are truly effectors is likely to be even lower. Furthermore, tandem repeats were implicated by bioinformatics as a possible means of delivery of multiple copies of effectors into a plant host. Only two of the 108 predicted tandem repeats were verified by transcriptomics, suggesting that tandem repeats are not likely to be an important effector mechanism in the *T. virens*-maize interaction. Further analysis of the proteins that were differentially expressed, showed only a small number were secreted molecules (**Figure 51**). The low number of secreted proteins detected suggests either that secreted effector molecules are not as important to endophytes as thought, or that a non-classical secretion signal exists. ELM searches for such a signal found three motifs in the differentially expressed proteins but these were only present in three proteins. The lack of a known fungal secretion signal is a major cause of the lack of predictive power (S. Zhang & Xu, 2014).

Some evidence for classical MAMP/PAMP recognition pathways was shown in maize. Differential expression of MAPK, WRKY and WAK proteins was identified in maize at 3 DPI. Other signalling proteins such as receptor kinases and NBS-LRR proteins were also differentially expressed. WRKY and WAK proteins are capable of initiating and modulating the response to invasion (Kanneganti & Gupta, 2008; Rushton et al., 2010). NBS-LRR proteins are the primary receptors thought to be involved in perception of MAMPs and effectors (McHale et al., 2006). Effectors may therefore be identifiable by disruption of maize defence genes, such as WRKY or WAK, which should affect the interaction. Alternatively, high throughput disruption of the unknown genes detected may also provide a means by which to identify effector proteins in *T. virens*.

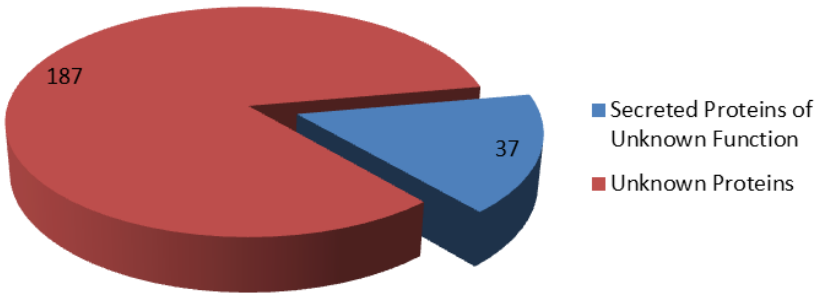


Figure 51: The proportion of differentially expressed unknown proteins that Contain a secretion signal Only 37 unknown proteins contained a known secretion signal, a hallmark of potential effector proteins.

Hormones and secondary metabolites formed another major group of up-regulated genes. These were produced by both *T. virens* and maize. *Trichoderma* secondary metabolites from a wide range of pathways were up-regulated. Most surprising was the presence of toxins, such as aflatoxin and trichocethenes in the transcriptome data. The up-regulation of toxins may suggest that toxicity towards the host is required for establishment of the interaction. *T. virens* also produced ACC-synthases which suggests that it may be mimicking ethylene hormone signalling. Ethylene signalling may play a role in colonisation and probably growth promotion (K. L.-C. Wang et al., 2002). Ethylene production may also be associated with increased susceptibility to fungal colonisation (Lund, Stall, & Klee, 1998; van Loon, Geraats, & Linthorst, 2006). Kynurenines were also produced by *T. virens* and may act alongside ethylene to modulate plant hormonal pathways (He et al., 2011; Wilson et al., 2003). Quinate related genes, such as quinate permeases may be linked to the CWDE functions, and facilitate transport of non-polysaccharide products of cell wall breakdown. Quinates are also associated with lignification of plant cell walls (Bhuiyan et al., 2009).

Sterols, terpenes and polyketides were also produced by *T. virens*. The diversity of possible products resulting from these pathways makes it difficult to comment on specific functions. However, sterols, terpenes and polyketides all have a range of activity ranging from antibiotic activity and pathogen resistance to precursors of hormones (Gräfe et al., 1991; Rodríguez et al., 2014; Weete et al., 2010). Polyketides in particular have been observed as Avr factors, hinting at a role in colonisation, at least for pathogens and impairment of their activity affects *T. virens* ability to modulate plant resistance (Degenkolb et al., 2008; Mukherjee, Horwitz, et al., 2012). Isolation and analysis of volatiles produced during the interaction may provide a means of identifying the exact function of compound produced. Numerous chromatography and extraction techniques exist that would serve this purpose (Bucar, Wube, & Schmid, 2013).

Maize appears to up-regulate a range of genes related to secondary metabolites in response to colonisation by *T. virens*. Ethylene, salicylic acid, gibberellic acid and jasmonic acid pathways were differentially expressed, though the evidence was less conclusive due to the generally lower resolution of transcriptomics results obtained in maize. Jasmonic acid, salicylic and gibberellic acid pathways are major defence related signalling pathways, and act antagonistically to determine the extent of the immune response, while ethylene is more traditionally associated with growth and wound responses (Davière & Achard, 2013; Lu, 2009; Shoresh et al., 2005; K. L.-C. Wang et al., 2002). Other trends in secondary metabolites production in maize appeared to be cell wall related,

particularly to do with lignin biosynthesis and deposition, which further supports the role of DAMP pathways in *T. virens* colonisation. Secondary metabolites are therefore likely to play numerous supporting roles in *T. virens* ability to colonise plants, and possibly in their ability to derive nutrition from their hosts. Isolation of compounds produced by *T. virens* and identification of the roles plant hormone pathways play in the interaction will be useful in isolating the role of secondary metabolites in root colonisation.

The final major group involved in the interaction was that of redox related genes. Peroxidase genes were up-regulated in maize across the interaction, and are generally responsible for generation of oxidative bursts. *Trichoderma virens* produced a range of metal-ion containing proteins, which have potential functions in signalling, iron scavenging and in redox tolerance. Peroxidases have been shown to form a major part of the PAMP response in *Arabidopsis* and the oxidative burst is a classical response to microbial invaders(Daudi et al., 2012). The production of redox related proteins in *T. harzianum* is associated with increased tolerance to reactive oxygen species in both the fungus and the host plant (Brahma N. Singh, 2011). This could implicate *T. virens* redox related genes in immune priming as well as root colonisation. Peroxidase activity is measurable with H_2O_2 assays, which may be capable of quantifying the effect of these genes in *T. virens* maize interactions.

7.4 Conclusion

Experimental confirmation of *T. virens* as a maize endophyte was obtained. The nature of the interaction appears to be specific, even within hybrid lines of the same host species (*Zea mays*). Despite effectors being predicted as a major factor in the interaction, CWDEs appeared to be the most consistently supported group of up-regulated genes, supported by polysaccharide transport. Secondary metabolites also appear to be important to *T. virens*-maize communication, although their role is more difficult to predict, due to the wide variety of such compounds produced. *T. virens* therefore appears to penetrate the host plant using CWDEs as well as physical mechanisms, and is capable of surviving as an endophyte for up to 7 DPI.

8 Appendix One: Transcriptomics of 12, 24 and 48 h Post Inoculation Interaction Between *T. virens* and *Z. mays* and Suppressive Hybridisation.

To examine the interactions at very early stages of root colonisation an RNA-sequencing experiment was carried out on the roots of plants grown under sterile conditions and then inoculated with *T. virens* after 3 days of growth. Root samples were taken at 12, 24 and 48 h post inoculation and total RNA was extracted from them. Total RNA was then run on the Illumina Hi-Seq system and analysed via the same pipeline as the DPI data in **Chapter 5**. Analysis of the data yielded highly variable results, with very low reads mapping to *T. virens* in most samples. BCV and dispersion values were very high, suggesting the data was unreliable, and this was clearly visualised in the volcano plots, which had numerous outliers. No clear trends could be identified in *T. virens* using this data. The maize data was more reliable than the fungal data, as the vast majority of reads had mapped to the maize genome. It was possible to identify some differentially expressed pathogenesis related genes, as well as jasmonic acid signalling pathways (**Table 33**). However, the vast majority of maize genes were not differentially expressed at this time point, suggesting that the number of maize cells interacting with *T. virens* was insufficient at this time point to provide useful data.

Table 33: Differentially expressed maize genes with immune related function – HPI datasets

Gene Identifier	Function
GRMZM2G104945	Defence response to fungus and systemic acquired resistance
GRMZM2G063306	Plant response to nematodes, trans membrane transport
GRMZM5G840145	Trehalose phosphatase, biosynthesis of trehalose
GRMZM2G337594	Wir1b like pathogenic resistance protein
GRMZM2G438824	Jasmonate-induced protein
GMZMM2G017629	Jasmonate-induced mRNA

These 6 differentially expressed maize genes were identified in the 12 HPI dataset. While it is hard to draw conclusive evidence from such limited data, it is interesting that 20.9% of the differentially expressed genes were related to immune signalling pathways.

Due to the lack of success in this experiment, it was decided to use the longer time points that correlated more strongly with levels of colonisation seen in the microscope data in **Chapter 3**. An attempt at performing suppressive hybridisation on the 3, 5 and 7 day samples was made due to the results of this experiment. The results indicated that read counts may be an issue in subsequent experiments. Suppressive hybridisation yielded good results for only one of the 3 samples trialled, and so this approach was abandoned. The later time points proved to have increased fungal content as per the microscopy results, and reads were sufficient for analysis without requiring suppressive hybridisation. Using unmodified total RNA for the analysis was thus decided upon. This also avoided possible sampling biases introduced by creation of cDNA libraries for suppressive hybridisation.

This early stage experiment did however allow improved methodology to be implemented and informed the selection of time points for the larger study discussed in **Chapter 5**. It also allowed analysis software issues to be isolated before the large scale study was performed. This led to use of CLC genomics workbench and EdgeR for future analysis.

9 References

Abbott, D. W., & Boraston, A. B. (2007). The structural basis for exopolygalacturonase activity in a family 28 glycoside hydrolase. *Journal of Molecular Biology*, *368*(5), 1215–1222. http://doi.org/10.1016/j.jmb.2007.02.083

Abubaker, K. S., Sjaarda, C., & Castle, A. J. (2013). Regulation of three genes encoding cell-wall-degrading enzymes of Trichoderma aggressivum during interaction with Agaricus bisporus. *Canadian Journal of Microbiology*, *59*(6), 417–424. http://doi.org/10.1139/cjm-2013-0173

Aguirre, J., Hansberg, W., & Navarro, R. (2006). Fungal responses to reactive oxygen species. *Medical Mycology*, *44*(Supplement 1), S101–S107. http://doi.org/10.1080/13693780600900080

Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y., Martin, J., Smart, L., … Ton, J. (2011). Benzoxazinoid Metabolites Regulate Innate Immunity against Aphids and Fungi in Maize1[W][OA]. *Plant Physiology*, *157*(1), 317–327. http://doi.org/10.1104/pp.111.180224

Alarcón, C. R., Lee, H., Goodarzi, H., Halberg, N., & Tavazoie, S. F. (2015). N6-methyladenosine marks primary microRNAs for processing. *Nature*, *519*(7544), 482–485. http://doi.org/10.1038/nature14281

Alfano, G., Ivey, M. L. L., Cakir, C., Bos, J. I. B., Miller, S. A., Madden, L. V., … Hoitink, H. a. J. (2007). Systemic Modulation of Gene Expression in Tomato by Trichoderma hamatum 382. *Phytopathology*, *97*(4), 429–437. http://doi.org/10.1094/PHYTO-97-4-0429

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. http://doi.org/10.1016/S0022-2836(05)80360-2

Annis, S. L., & Goodwin, P. H. (1997). Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *European Journal of Plant Pathology*, *103*(1), 1–14. http://doi.org/10.1023/A:1008656013255

Arnaud, D., & Hwang, I. (2015). A Sophisticated Network of Signaling Pathways Regulates Stomatal Defenses to Bacterial Pathogens. *Molecular Plant*, *8*(4), 566–581. http://doi.org/10.1016/j.molp.2014.10.012

Arnold, R., Brandmaier, S., Kleine, F., Tischler, P., Heinz, E., Behrens, S., … Rattei, T. (2009). Sequence-based prediction of type III secreted proteins. *PLoS Pathogens*, *5*(4), e1000376. http://doi.org/10.1371/journal.ppat.1000376

Aro, N., Saloheimo, A., Ilmén, M., & Penttilä, M. (2001). ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of Trichoderma reesei. *The Journal of Biological Chemistry*, *276*(26), 24309–24314. http://doi.org/10.1074/jbc.M003624200

Atsatt, P. R., & Whiteside, M. D. (2014). Novel Symbiotic Protoplasts Formed by Endophytic Fungi Explain Their Hidden Existence, Lifestyle Switching, and Diversity within the Plant Kingdom. *PLoS ONE*, *9*(4), e95266. http://doi.org/10.1371/journal.pone.0095266

Avni, A., Bailey, B. A., Mattoo, A. K., & Anderson, J. D. (1994). Induction of Ethylene Biosynthesis in Nicotiana tabacum by a Trichoderma viride Xylanase Is Correlated to the Accumulation of 1-Aminocyclopropane-1-Carboxylic Acid (ACC) Synthase and ACC Oxidase Transcripts. *Plant Physiology*, *106*(3), 1049–1055. http://doi.org/10.1104/pp.106.3.1049

Ayala, J. C., Wang, H., Benitez, J. A., & Silva, A. J. (2015). RNA-Seq analysis and whole genome DNA-binding profile of the Vibrio cholerae histone-like nucleoid structuring protein (H-NS). *Genomics Data*, *5*, 147–150. http://doi.org/10.1016/j.gdata.2015.05.039

Babu, A. G., Shea, P. J., & Oh, B.-T. (2014). Trichoderma sp. PDR1-7 promotes Pinus sylvestris reforestation of lead-contaminated mine tailing sites. *The Science of the Total Environment*, *476-477*, 561–567. http://doi.org/10.1016/j.scitotenv.2013.12.119

Babu, A. G., Shim, J., Bang, K.-S., Shea, P. J., & Oh, B.-T. (2014). Trichoderma virens PDR-28: a heavy metal-tolerant and plant growth-promoting fungus for remediation and bioenergy crop production on mine tailing soil. *Journal of Environmental Management*, *132*, 129–134. http://doi.org/10.1016/j.jenvman.2013.10.009

Bacic, A., Moody, S. F., & Clarke, A. E. (1986). Structural Analysis of Secreted Root Slime from Maize (Zea mays L.). *Plant Physiology*, *80*(3), 771–777.

Bae, H., Bowers, J. H., Tooley, P. W., & Bailey, B. A. (2005). NEP1 orthologs encoding necrosis and ethylene inducing proteins exist as a multigene family in Phytophthora megakarya, causal agent of black pod disease on cacao. *Mycological Research*, *109*(Pt 12), 1373–1385.

Bae, H., Kim, M. S., Sicher, R. C., Bae, H.-J., & Bailey, B. A. (2006). Necrosis- and ethylene-inducing peptide from Fusarium oxysporum induces a complex cascade of transcripts associated with signal transduction and cell death in Arabidopsis. *Plant Physiology*, *141*(3), 1056–1067. http://doi.org/10.1104/pp.106.076869

Baek, J. M., & Kenerley, C. M. (1998). The arg2 gene of Trichoderma virens: cloning and development of a homologous transformation system. *Fungal Genetics and Biology: FG & B*, *23*(1), 34–44. http://doi.org/10.1006/fgbi.1997.1025

Bailey, B. A., Bae, H., Strem, M. D., Roberts, D. P., Thomas, S. E., Crozier, J., … Holmes, K. A. (2006). Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four Trichoderma species. *Planta*, *224*(6), 1449–1464. http://doi.org/10.1007/s00425-006-0314-0

Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E., Clementi, L., … Noble, W. S. (2009). MEME Suite: tools for motif discovery and searching. *Nucleic Acids Research*, *37*(suppl 2), W202–W208. http://doi.org/10.1093/nar/gkp335

Bakker, P. A. H. M., Berendsen, R. L., Doornbos, R. F., Wintermans, P. C. A., & Pieterse, C. M. J. (2013). The rhizosphere revisited: root microbiomics. *Frontiers in Plant Science*, *4*. http://doi.org/10.3389/fpls.2013.00165

Bar, M., Sharfman, M., Ron, M., & Avni, A. (2010). BAK1 is required for the attenuation of ethylene-inducing xylanase (Eix)-induced defense responses by the decoy receptor LeEix1. *The Plant Journal: For Cell and Molecular Biology*, *63*(5), 791–800. http://doi.org/10.1111/j.1365-313X.2010.04282.x

Barnett, J. P., Eijlander, R. T., Kuipers, O. P., & Robinson, C. (2008). A minimal Tat system from a gram-positive organism: a bifunctional TatA subunit participates in discrete TatAC and TatA complexes. *The Journal of Biological Chemistry*, *283*(5), 2534–2542. http://doi.org/10.1074/jbc.M708134200

Beliën, T., Van Campenhout, S., Robben, J., & Volckaert, G. (2006). Microbial Endoxylanases: Effective Weapons to Breach the Plant Cell-Wall Barrier or, Rather, Triggers of Plant Defense Systems? *Molecular Plant-Microbe Interactions*, *19*(10), 1072–1081. http://doi.org/10.1094/MPMI-19-1072

Bellincampi, D., Cervone, F., & Lionetti, V. (2014). Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. *Frontiers in Plant Science*, *5*, 228. http://doi.org/10.3389/fpls.2014.00228

Benedetti, M., Pontiggia, D., Raggi, S., Cheng, Z., Scaloni, F., Ferrari, S., … Lorenzo, G. D. (2015). Plant immunity triggered by engineered in vivo release of oligogalacturonides, damage-

associated molecular patterns. *Proceedings of the National Academy of Sciences*, *112*(17), 5533–5538. http://doi.org/10.1073/pnas.1504154112

Bent, A. F., & Mackey, D. (2007). Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annual Review of Phytopathology*, *45*, 399–436. http://doi.org/10.1146/annurev.phyto.45.062806.094427

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G., … Smith, A. J. (2008). Accurate whole human genome sequencing using reversible terminator chemistry. *Nature*, *456*(7218), 53–59. http://doi.org/10.1038/nature07517

Bhattacharya, A., Sood, P., & Citovsky, V. (2010). The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection. *Molecular Plant Pathology*, *11*(5), 705–719. http://doi.org/10.1111/j.1364-3703.2010.00625.x

Bhuiyan, N. H., Selvaraj, G., Wei, Y., & King, J. (2009). Role of lignification in plant defense. *Plant Signaling & Behavior*, *4*(2), 158–159.

Birch, P. R. J., Armstrong, M., Bos, J., Boevink, P., Gilroy, E. M., Taylor, R. M., … Kamoun, S. (2009). Towards understanding the virulence functions of RXLR effectors of the oomycete plant pathogen Phytophthora infestans. *Journal of Experimental Botany*, *60*(4), 1133–1140. http://doi.org/10.1093/jxb/ern353

Bittel, P., & Robatzek, S. (2007). Microbe-associated molecular patterns (MAMPs) probe plant immunity. *Current Opinion in Plant Biology*, *10*(4), 335–341. http://doi.org/10.1016/j.pbi.2007.04.021

Boch, J., & Bonas, U. (2010). Xanthomonas AvrBs3 family-type III effectors: discovery and function. *Annual Review of Phytopathology*, *48*, 419–436. http://doi.org/10.1146/annurev-phyto-080508-081936

Bohnert, H. U., Fudal, I., Dioh, W., Tharreau, D., Notteghem, J.-L., & Lebrun, M.-H. (2004). A Putative Polyketide Synthase/Peptide Synthetase from Magnaporthe grisea Signals Pathogen Attack to Resistant Rice. *The Plant Cell*, *16*(9), 2499–2513. http://doi.org/10.1105/tpc.104.022715

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina Sequence Data. *Bioinformatics*, btu170. http://doi.org/10.1093/bioinformatics/btu170

Boller, T., & Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology*, *60*, 379–406. http://doi.org/10.1146/annurev.arplant.57.032905.105346

Bolouri-Moghaddam, M. R., Le Roy, K., Xiang, L., Rolland, F., & Van den Ende, W. (2010). Sugar signalling and antioxidant network connections in plant cells. *The FEBS Journal*, *277*(9), 2022–2037. http://doi.org/10.1111/j.1742-4658.2010.07633.x

Bonfante, P., & Requena, N. (2011). Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology*, *14*(4), 451–457. http://doi.org/10.1016/j.pbi.2011.03.014

Bordiec, S., Paquis, S., Lacroix, H., Dhondt, S., Ait Barka, E., Kauffmann, S., … Dorey, S. (2011). Comparative analysis of defence responses induced by the endophytic plant growth-promoting rhizobacterium Burkholderia phytofirmans strain PsJN and the non-host bacterium Pseudomonas syringae pv. pisi in grapevine cell suspensions. *Journal of Experimental Botany*, *62*(2), 595–603. http://doi.org/10.1093/jxb/erq291

Bozkurt, T. O., Schornack, S., Banfield, M. J., & Kamoun, S. (2012). Oomycetes, effectors, and all that jazz. *Current Opinion in Plant Biology*, *15*(4), 483–492. http://doi.org/10.1016/j.pbi.2012.03.008

Brahma N. Singh, A. S. (2011). Trichoderma harzianum- mediated reprogramming of oxidative stress response in root apoplast of sunflower enhances defence against Rhizoctonia solani.

European Journal of Plant Pathology, *131*(1), 121–134. http://doi.org/10.1007/s10658-011-9792-4

Brotman, Y., Briff, E., Viterbo, A., & Chet, I. (2008). Role of Swollenin, an Expansin-Like Protein from Trichoderma, in Plant Root Colonization. *Plant Physiology*, *147*(2), 779–789. http://doi.org/10.1104/pp.108.116293

Brotman, Y., Landau, U., Cuadros-Inostroza, A., Takayuki, T., Fernie, A. R., Chet, I., … Willmitzer, L. (2013). Trichoderma-Plant Root Colonization: Escaping Early Plant Defense Responses and Activation of the Antioxidant Machinery for Saline Stress Tolerance. *PLoS Pathogens*, *9*(3). http://doi.org/10.1371/journal.ppat.1003221

Bucar, F., Wube, A., & Schmid, M. (2013). Natural product isolation--how to get from biological material to pure compounds. *Natural Product Reports*, *30*(4), 525–545. http://doi.org/10.1039/c3np20106f

Burstein, D., Zusman, T., Degtyar, E., Viner, R., Segal, G., & Pupko, T. (2009). Genome-scale identification of Legionella pneumophila effectors using a machine learning approach. *PLoS Pathogens*, *5*(7), e1000508. http://doi.org/10.1371/journal.ppat.1000508

Bush, L. P., Wilkinson, H. H., & Schardl, C. L. (1997). Bioprotective Alkaloids of Grass-Fungal Endophyte Symbioses. *Plant Physiology*, *114*(1), 1–7.

Cai, X.-Z., Xu, Y.-P., & Zheng, Z. (2002). [Avirulence genes of plant pathogens]. *Sheng Wu Gong Cheng Xue Bao = Chinese Journal of Biotechnology*, *18*(1), 5–9.

Cannon, P. F., & Simmons, C. M. (2002). Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. *Mycologia*, *94*(2), 210–220.

Caño-Delgado, A., Penfield, S., Smith, C., Catley, M., & Bevan, M. (2003). Reduced cellulose synthesis invokes lignification and defense responses in Arabidopsis thaliana. *The Plant Journal: For Cell and Molecular Biology*, *34*(3), 351–362.

Carapito, R., Hatsch, D., Vorwerk, S., Petkovski, E., Jeltsch, J.-M., & Phalip, V. (2008). Gene expression in Fusarium graminearum grown on plant cell wall. *Fungal Genetics and Biology*, *45*(5), 738–748. http://doi.org/10.1016/j.fgb.2007.12.002

Catanzariti, A.-M., Dodds, P. N., & Ellis, J. G. (2007). Avirulence proteins from haustoria-forming pathogens. *FEMS Microbiology Letters*, *269*(2), 181–188. http://doi.org/10.1111/j.1574-6968.2007.00684.x

Chacón, M. R., Rodríguez-Galán, O., Benítez, T., Sousa, S., Rey, M., Llobell, A., & Delgado-Jarana, J. (2007). Microscopic and transcriptome analyses of early colonization of tomato roots by Trichoderma harzianum. *International Microbiology: The Official Journal of the Spanish Society for Microbiology*, *10*(1), 19–27.

Chandler, D., Bailey, A. S., Tatchell, G. M., Davidson, G., Greaves, J., & Grant, W. P. (2011). The development, regulation and use of biopesticides for integrated pest management. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *366*(1573), 1987–1998. http://doi.org/10.1098/rstb.2010.0390

Charlton, N. D., Shoji, J.-Y., Ghimire, S. R., Nakashima, J., & Craven, K. D. (2012). Deletion of the Fungal Gene soft Disrupts Mutualistic Symbiosis between the Grass Endophyte Epichloë festucae and the Host Plant. *Eukaryotic Cell*, *11*(12), 1463–1471. http://doi.org/10.1128/EC.00191-12

Choi, J., Park, J., Kim, D., Jung, K., Kang, S., & Lee, Y.-H. (2010). Fungal secretome database: integrated platform for annotation of fungal secretomes. *BMC Genomics*, *11*, 105. http://doi.org/10.1186/1471-2164-11-105

Chu, K. T., & Ng, T. B. (2003). Isolation of a large thaumatin-like antifungal protein from seeds of the Kweilin chestnut Castanopsis chinensis. *Biochemical and Biophysical Research Communications*, *301*(2), 364–370.

Coburn, B., Sekirov, I., & Finlay, B. B. (2007). Type III Secretion Systems and Disease. *Clinical Microbiology Reviews*, *20*(4), 535–549. http://doi.org/10.1128/CMR.00013-07

Colburn-Clifford, J., & Allen, C. (2010). A cbb(3)-type cytochrome C oxidase contributes to Ralstonia solanacearum R3bv2 growth in microaerobic environments and to bacterial wilt disease development in tomato. *Molecular Plant-Microbe Interactions: MPMI*, *23*(8), 1042–1052. http://doi.org/10.1094/MPMI-23-8-1042

Conesa. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, *21*.

Conesa, A., & Gotz, S. (2008). Blast2GO: A Comprehensive Suite for Functional Analysis in Plant Genomics. *International Journal of Plant Genomics*, *2008*. http://doi.org/10.1155/2008/619832

Cornelissen, B. J., Hooft van Huijsduijnen, R. A., & Bol, J. F. (1986). A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. *Nature*, *321*(6069), 531–532. http://doi.org/10.1038/321531a0

Cosgrove, D. J. (2000). Loosening of plant cell walls by expansins. *Nature*, *407*(6802), 321–326. http://doi.org/10.1038/35030000

Costa, T. R. D., Felisberto-Rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M., & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nature Reviews Microbiology*, *13*(6), 343–359. http://doi.org/10.1038/nrmicro3456

Cripps-Guazzone, N. (2014). *Rhizosphere competence of selected Trichoderma species* (Thesis). Lincoln University. Retrieved from https://researcharchive.lincoln.ac.nz/handle/10182/6465

Cui, H., Tsuda, K., & Parker, J. E. (2015). Effector-Triggered Immunity: From Pathogen Perception to Robust Defense. *Annual Review of Plant Biology*, *66*(1), 487–511. http://doi.org/10.1146/annurev-arplant-050213-040012

Dagdas, Y. F., Yoshino, K., Dagdas, G., Ryder, L. S., Bielska, E., Steinberg, G., & Talbot, N. J. (2012). Septin-Mediated Plant Cell Invasion by the Rice Blast Fungus, Magnaporthe oryzae. *Science*, *336*(6088), 1590–1595. http://doi.org/10.1126/science.1222934

Dashtban, M., Schraft, H., Syed, T. A., & Qin, W. (2010). Fungal biodegradation and enzymatic modification of lignin. *International Journal of Biochemistry and Molecular Biology*, *1*(1), 36–50.

Daudi, A., Cheng, Z., O'Brien, J. A., Mammarella, N., Khan, S., Ausubel, F. M., & Bolwell, G. P. (2012). The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pattern-triggered immunity. *The Plant Cell*, *24*(1), 275–287. http://doi.org/10.1105/tpc.111.093039

Davière, J.-M., & Achard, P. (2013). Gibberellin signaling in plants. *Development*, *140*(6), 1147–1151. http://doi.org/10.1242/dev.087650

Dean, P. (2011). Functional domains and motifs of bacterial type III effector proteins and their roles in infection. *FEMS Microbiology Reviews*, *35*(6), 1100–1125. http://doi.org/10.1111/j.1574-6976.2011.00271.x

Dean, P., & Kenny, B. (2009). The effector repertoire of enteropathogenic E. coli: ganging up on the host cell. *Current Opinion in Microbiology*, *12*(1), 101–109. http://doi.org/10.1016/j.mib.2008.11.006

Degenkolb, T., von Döhren, H., Nielsen, K. F., Samuels, G. J., & Brückner, H. (2008). Recent advances and future prospects in peptaibiotics, hydrophobin, and mycotoxin research, and their importance for chemotaxonomy of Trichoderma and Hypocrea. *Chemistry & Biodiversity*, *5*(5), 671–680. http://doi.org/10.1002/cbdv.200890064

Degrassi, G., Okeke, B. C., Bruschi, C. V., & Venturi, V. (1998). Purification and Characterization of an Acetyl Xylan Esterase from Bacillus pumilus. *Applied and Environmental Microbiology*, *64*(2), 789–792.

de Guillen, K., Ortiz-Vallejo, D., Gracy, J., Fournier, E., Kroj, T., & Padilla, A. (2015). Structure Analysis Uncovers a Highly Diverse but Structurally Conserved Effector Family in Phytopathogenic Fungi. *PLoS Pathog*, *11*(10), e1005228. http://doi.org/10.1371/journal.ppat.1005228

Deising, H. B., Werner, S., & Wernitz, M. (2000). The role of fungal appressoria in plant infection. *Microbes and Infection / Institut Pasteur*, *2*(13), 1631–1641.

De Jaeger, N., Declerck, S., & de la Providencia, I. E. (2010). Mycoparasitism of arbuscular mycorrhizal fungi: a pathway for the entry of saprotrophic fungi into roots. *FEMS Microbiology Ecology*, *73*(2), 312–322. http://doi.org/10.1111/j.1574-6941.2010.00903.x

de Jonge, R., & Thomma, B. P. H. J. (2009). Fungal LysM effectors: extinguishers of host immunity? *Trends in Microbiology*, *17*(4), 151–157. http://doi.org/10.1016/j.tim.2009.01.002

de Jonge, R., van Esse, H. P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., … Thomma, B. P. H. J. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science (New York, N.Y.)*, *329*(5994), 953–955. http://doi.org/10.1126/science.1190859

Delaye, L., García-Guzmán, G., & Heil, M. (2013). Endophytes versus biotrophic and necrotrophic pathogens—are fungal lifestyles evolutionarily stable traits? *Fungal Diversity*, *60*(1), 125–135. http://doi.org/10.1007/s13225-013-0240-y

Denness, L., McKenna, J. F., Segonzac, C., Wormit, A., Madhou, P., Bennett, M., … Hamann, T. (2011). Cell wall damage-induced lignin biosynthesis is regulated by a reactive oxygen species- and jasmonic acid-dependent process in Arabidopsis. *Plant Physiology*, *156*(3), 1364–1374. http://doi.org/10.1104/pp.111.175737

de Souza, W. R., Maitan-Alfenas, G. P., de Gouvêa, P. F., Brown, N. A., Savoldi, M., Battaglia, E., … Goldman, G. H. (2013). The influence of Aspergillus niger transcription factors AraR and XlnR in the gene expression during growth in d-xylose, l-arabinose and steam-exploded sugarcane bagasse. *Fungal Genetics and Biology*, *60*, 29–45. http://doi.org/10.1016/j.fgb.2013.07.007

De Wit, P. J. G. M., Mehrabi, R., Van Den Burg, H. A., & Stergiopoulos, I. (2009). Fungal effector proteins: past, present and future. *Molecular Plant Pathology*, *10*(6), 735–747. http://doi.org/10.1111/j.1364-3703.2009.00591.x

Dickman, M. B., & de Figueiredo, P. (2013). Death Be Not Proud—Cell Death Control in Plant Fungal Interactions. *PLoS Pathog*, *9*(9), e1003542. http://doi.org/10.1371/journal.ppat.1003542

Djamei, A., & Kahmann, R. (2012). Ustilago maydis: Dissecting the Molecular Interface between Pathogen and Plant. *PLoS Pathog*, *8*(11), e1002955. http://doi.org/10.1371/journal.ppat.1002955

Djonović, S., Pozo, M. J., Dangott, L. J., Howell, C. R., & Kenerley, C. M. (2006). Sm1, a proteinaceous elicitor secreted by the biocontrol fungus Trichoderma virens induces plant defense responses and systemic resistance. *Molecular Plant-Microbe Interactions: MPMI*, *19*(8), 838–853. http://doi.org/10.1094/MPMI-19-0838

Djonović, S., Pozo, M. J., & Kenerley, C. M. (2006). Tvbgn3, a beta-1,6-glucanase from the biocontrol fungus Trichoderma virens, is involved in mycoparasitism and control of Pythium ultimum. *Applied and Environmental Microbiology*, *72*(12), 7661–7670. http://doi.org/10.1128/AEM.01607-06

Djonovic, S., Vargas, W. A., Kolomiets, M. V., Horndeski, M., Wiest, A., & Kenerley, C. M. (2007). A proteinaceous elicitor Sm1 from the beneficial fungus Trichoderma virens is required for

induced systemic resistance in maize. *Plant Physiology*, *145*(3), 875–889. http://doi.org/10.1104/pp.107.103689

Dodds, P. N., Lawrence, G. J., Catanzariti, A.-M., Teh, T., Wang, C.-I. A., Ayliffe, M. A., … Ellis, J. G. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(23), 8888–8893. http://doi.org/10.1073/pnas.0602577103

Doehlemann, G., van der Linde, K., Assmann, D., Schwammbach, D., Hof, A., Mohanty, A., … Kahmann, R. (2009). Pep1, a secreted effector protein of Ustilago maydis, is required for successful invasion of plant cells. *PLoS Pathogens*, *5*(2), e1000290. http://doi.org/10.1371/journal.ppat.1000290

Dong, S., Kong, G., Qutob, D., Yu, X., Tang, J., Kang, J., … Wang, Y. (2012). The NLP toxin family in Phytophthora sojae includes rapidly evolving groups that lack necrosis-inducing activity. *Molecular Plant-Microbe Interactions: MPMI*, *25*(7), 896–909. http://doi.org/10.1094/MPMI-01-12-0023-R

Dong, S., Yin, W., Kong, G., Yang, X., Qutob, D., Chen, Q., … Wang, Y. (2011). Phytophthora sojae avirulence effector Avr3b is a secreted NADH and ADP-ribose pyrophosphorylase that modulates plant immunity. *PLoS Pathogens*, *7*(11), e1002353. http://doi.org/10.1371/journal.ppat.1002353

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., … Kubicek, C. P. (2011). Trichoderma: the genomics of opportunistic success. *Nature Reviews Microbiology*, *9*(10), 749–759. http://doi.org/10.1038/nrmicro2637

Dubey, M. K., Jensen, D. F., & Karlsson, M. (2014). Hydrophobins are required for conidial hydrophobicity and plant root colonization in the fungal biocontrol agent Clonostachys rosea. *BMC Microbiology*, *14*(1), 18. http://doi.org/10.1186/1471-2180-14-18

Eaton, C. J., Cox, M. P., & Scott, B. (2011). What triggers grass endophytes to switch from mutualism to pathogenism? *Plant Science*, *180*(2), 190–195. http://doi.org/10.1016/j.plantsci.2010.10.002

Eaton, C. J., Dupont, P.-Y., Solomon, P., Clayton, W., Scott, B., & Cox, M. P. (2014). A Core Gene Set Describes the Molecular Basis of Mutualism and Antagonism in Epichloë spp. *Molecular Plant-Microbe Interactions*, *28*(3), 218–231. http://doi.org/10.1094/MPMI-09-14-0293-FI

Elad, Y., Chet, I., & Henis, Y. (1981). A selective medium for improving quantitative isolation ofTrichoderma spp. from soil. *Phytoparasitica*, *9*(1), 59–67. http://doi.org/10.1007/BF03158330

Ellinger, D., Naumann, M., Falter, C., Zwikowics, C., Jamrow, T., Manisseri, C., … Voigt, C. A. (2013). Elevated early callose deposition results in complete penetration resistance to powdery mildew in Arabidopsis. *Plant Physiology*, *161*(3), 1433–1444. http://doi.org/10.1104/pp.112.211011

Uzcategui, E., Ruiz, A., Monstesino, R., Johansson, G.,& Pettersson, G. (1991). The 1,4-beta-D-glucan cellobiohydrolases from Phanerochaete chrysosporium. I. A system of synergistically acting enzymes homologous to Trichoderma reesei. *Journal of Biotechnology*, *19*(2-3), 271–285. http://doi.org/10.1016/0168-1656(91)90064-3

Eveland, A. L., & Jackson, D. P. (2011). Sugars, signalling, and plant development. *Journal of Experimental Botany*, err379. http://doi.org/10.1093/jxb/err379

Facchini, P. J. (2001). Facchini, P. J. Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. Annu. Rev. Plant Physiol.

Plant Mol. Biol. 52, 29-66. *Annual Review of Plant Physiology and Plant Molecular Biology*, *52*, 29–66. http://doi.org/10.1146/annurev.arplant.52.1.29

Feechan, A., Turnbull, D., Stevens, L. J., Engelhardt, S., Birch, P. R. J., Hein, I., & Gilroy, E. M. (2015). The Hypersensitive Response in PAMP- and Effector-Triggered Immune Responses. In A. N. Gunawardena & P. F. McCabe (Eds.), *Plant Programmed Cell Death* (pp. 235–268). Springer International Publishing. Retrieved from http://link.springer.com/chapter/10.1007/978-3-319-21033-9_10

Felse, P. A., & Panda, T. (1999). Regulation and cloning of microbial chitinase genes. *Applied Microbiology and Biotechnology*, *51*(2), 141–151.

Fincham, J. R. (1989). Transformation in fungi. *Microbiological Reviews*, *53*(1), 148–170.

Frankie K. Crutcher, M. E. M.-D. (2015). A paralog of the proteinaceous elicitor SM1 is involved in colonization of maize roots by Trichoderma virens. *Fungal Biology*, *119*(6). http://doi.org/10.1016/j.funbio.2015.01.004

F. Vinale, K. S. (2008). A novel role for Trichoderma secondary metabolites in the interactions with plants. *Physiological and Molecular Plant Pathology*, 80–86. http://doi.org/10.1016/j.pmpp.2008.05.005

Gaderer, R., Lamdan, N. L., Frischmann, A., Sulyok, M., Krska, R., Horwitz, B. A., & Seidl-Seiboth, V. (2015). Sm2, a paralog of the Trichoderma cerato-platanin elicitor Sm1, is also highly important for plant protection conferred by the fungal-root interaction of Trichoderma with maize. *BMC Microbiology*, *15*(1), 1–9. http://doi.org/10.1186/s12866-014-0333-0

Gao, Q.-M., Zhu, S., Kachroo, P., & Kachroo, A. (2015). Signal regulators of systemic acquired resistance. *Frontiers in Plant Science*, *6*. http://doi.org/10.3389/fpls.2015.00228

Gassmann, W., & Bhattacharjee, S. (2012). Effector-Triggered Immunity Signaling: From Gene-for-Gene Pathways to Protein-Protein Interaction Networks. *Molecular Plant-Microbe Interactions*, *25*(7), 862–868. http://doi.org/10.1094/MPMI-01-12-0024-IA

Geldner, N. (2004). The plant endosomal system—its structure and role in signal transduction and plant development. *Planta*, *219*(4), 547–560. http://doi.org/10.1007/s00425-004-1302-x

Germaine, K., Keogh, E., Garcia-Cabellos, G., Borremans, B., van der Lelie, D., Barac, T., … Dowling, D. N. (2004). Colonisation of poplar trees by gfp expressing bacterial endophytes. *FEMS Microbiology Ecology*, *48*(1), 109–118. http://doi.org/10.1016/j.femsec.2003.12.009

Gilbert, H. J. (2010). The Biochemistry and Structural Biology of Plant Cell Wall Deconstruction. *Plant Physiology*, *153*(2), 444–455. http://doi.org/10.1104/pp.110.156646

Giraldo, M. C., Dagdas, Y. F., Gupta, Y. K., Mentlak, T. A., Yi, M., Martinez-Rocha, A. L., … Valent, B. (2013). Two distinct secretion systems facilitate tissue invasion by the rice blast fungus Magnaporthe oryzae. *Nature Communications*, *4*. http://doi.org/10.1038/ncomms2996

Giraldo, M. C., & Valent, B. (2013). Filamentous plant pathogen effectors in action. *Nature Reviews Microbiology*, *11*(11), 800–814. http://doi.org/10.1038/nrmicro3119

Godfrey, D., Böhlenius, H., Pedersen, C., Zhang, Z., Emmersen, J., & Thordal-Christensen, H. (2010). Powdery mildew fungal effector candidates share N-terminal Y/F/WxC-motif. *BMC Genomics*, *11*, 317. http://doi.org/10.1186/1471-2164-11-317

Göhre, V., & Robatzek, S. (2008). Breaking the barriers: microbial effector molecules subvert plant immunity. *Annual Review of Phytopathology*, *46*, 189–215. http://doi.org/10.1146/annurev.phyto.46.120407.110050

Gómez-Gómez, L., & Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell*, *5*(6), 1003–1011.

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., … Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research*, *36*(10), 3420–3435. http://doi.org/10.1093/nar/gkn176

Gräfe, U., Ihn, W., Schlegel, B., Höfle, G., Augustiniak, H., & Sandor, P. (1991). Structure of ergokonin C, a new carboxysterol antifungal antibiotic from a Tolypocladium inflatum mutant. *Die Pharmazie*, *46*(8), 613–614.

Greenshields, D. L., Liu, G., & Wei, Y. (2007). Roles of Iron in Plant Defence and Fungal Virulence. *Plant Signaling & Behavior*, *2*(4), 300–302.

Gregg, K. J. (2011). *Carbohydrate processing by bacterial pathogens: structural and functional analyses of glycoside hydrolases.* (Thesis). Retrieved from https://dspace.library.uvic.ca//handle/1828/3726

Gruber, S., & Seidl-Seiboth, V. (2012). Self versus non-self: fungal cell wall degradation in Trichoderma. *Microbiology (Reading, England)*, *158*(Pt 1), 26–34. http://doi.org/10.1099/mic.0.052613-0

Gudesblat, G. E., Torres, P. S., & Vojnov, A. A. (2009). Stomata and pathogens. *Plant Signaling & Behavior*, *4*(12), 1114–1116.

Gupta, S. K., Rai, A. K., Kanwar, S. S., & Sharma, T. R. (2012). Comparative Analysis of Zinc Finger Proteins Involved in Plant Disease Resistance. *PLoS ONE*, *7*(8), e42578. http://doi.org/10.1371/journal.pone.0042578

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., … Regev, A. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols*, *8*(8), 1494–1512. http://doi.org/10.1038/nprot.2013.084

Hajri, A., Brin, C., Hunault, G., Lardeux, F., Lemaire, C., Manceau, C., … Poussier, S. (2009). A "repertoire for repertoire" hypothesis: repertoires of type three effectors are candidate determinants of host specificity in Xanthomonas. *PloS One*, *4*(8), e6632. http://doi.org/10.1371/journal.pone.0006632

Hakkinen, M., Valkonen, M. J., Westerholm-Parvinen, A., Aro, N., Arvas, M., Vitikainen, M., … Pakula, T. M. (2014). Screening of candidate regulators for cellulase and hemicellulase production in Trichoderma reesei and identification of a factor essential for cellulase production. *Biotechnology for Biofuels*, *7*, 14. http://doi.org/10.1186/1754-6834-7-14

Halim, V. A., Vess, A., Scheel, D., & Rosahl, S. (2006). The role of salicylic acid and jasmonic acid in pathogen defence. *Plant Biology (Stuttgart, Germany)*, *8*(3), 307–313. http://doi.org/10.1055/s-2006-924025

Hanania, U., Furman-Matarasso, N., Ron, M., & Avni, A. (1999). Isolation of a novel SUMO protein from tomato that suppresses EIX-induced cell death. *The Plant Journal: For Cell and Molecular Biology*, *19*(5), 533–541.

Hann, C. T., Bequette, C. J., Dombrowski, J. E., & Stratmann, J. W. (2014). Methanol and ethanol modulate responses to danger- and microbe-associated molecular patterns. *Frontiers in Plant Science*, *5*. http://doi.org/10.3389/fpls.2014.00550

Hao, L.-Y., Willis, D. K., Andrews-Polymenis, H., McClelland, M., & Barak, J. D. (2012). Requirement of siderophore biosynthesis for plant colonization by Salmonella enterica. *Applied and Environmental Microbiology*, *78*(13), 4561–4570. http://doi.org/10.1128/AEM.07867-11

Harel, A., Bromberg, Y., Falkowski, P. G., & Bhattacharya, D. (2014). Evolutionary history of redox metal-binding domains across the tree of life. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(19), 7042–7047. http://doi.org/10.1073/pnas.1403676111

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004a). Trichoderma species--opportunistic, avirulent plant symbionts. *Nature Reviews. Microbiology*, *2*(1), 43–56. http://doi.org/10.1038/nrmicro797

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004b). Trichoderma species--opportunistic, avirulent plant symbionts. *Nature Reviews. Microbiology*, *2*(1), 43–56. http://doi.org/10.1038/nrmicro797

Hartmann, A., Rothballer, M., & Schmid, M. (2007). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant and Soil*, *312*(1-2), 7–14. http://doi.org/10.1007/s11104-007-9514-z

Hatsch, D., Phalip, V., Petkovski, E., & Jeltsch, J.-M. (2006). Fusarium graminearum on plant cell wall: No fewer than 30 xylanase genes transcribed. *Biochemical and Biophysical Research Communications*, *345*(3), 959–966. http://doi.org/10.1016/j.bbrc.2006.04.171

Hayashi, K., Takehisa, T., Hamato, N., Takano, R., Hara, S., Miyata, T., & Kato, H. (1994). Inhibition of serine proteases of the blood coagulation system by squash family protease inhibitors. *Journal of Biochemistry*, *116*(5), 1013–1018.

Heil, M., & Land, W. G. (2014). Danger signals – damaged-self recognition across the tree of life. *Frontiers in Plant Science*, *5*. http://doi.org/10.3389/fpls.2014.00578

Hématy, K., Cherk, C., & Somerville, S. (2009). Host-pathogen warfare at the plant cell wall. *Current Opinion in Plant Biology*, *12*(4), 406–413. http://doi.org/10.1016/j.pbi.2009.06.007

Hermosa, R., Viterbo, A., Chet, I., & Monte, E. (2012). Plant-beneficial effects of Trichoderma and of its genes. *Microbiology*, *158*(1), 17–25. http://doi.org/10.1099/mic.0.052274-0

Herrmann, K. M. (1995). The Shikimate Pathway: Early Steps in the Biosynthesis of Aromatic Compounds. *The Plant Cell*, *7*(7), 907–919. http://doi.org/10.1105/tpc.7.7.907

He, W., Brumos, J., Li, H., Ji, Y., Ke, M., Gong, X., … Guo, H. (2011). A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in Arabidopsis. *The Plant Cell*, *23*(11), 3944–3960. http://doi.org/10.1105/tpc.111.089029

Hilbert, M., Nostadt, R., & Zuccaro, A. (2013). Exogenous auxin affects the oxidative burst in barley roots colonized by Piriformospora indica. *Plant Signaling & Behavior*, *8*(4). http://doi.org/10.4161/psb.23572

Hogenhout, S. A., Van der Hoorn, R. A. L., Terauchi, R., & Kamoun, S. (2009). Emerging concepts in effector biology of plant-associated organisms. *Molecular Plant-Microbe Interactions: MPMI*, *22*(2), 115–122. http://doi.org/10.1094/MPMI-22-2-0115

Hong, S.-Y., Roze, L. V., & Linz, J. E. (2013). Oxidative stress-related transcription factors in the regulation of secondary metabolism. *Toxins*, *5*(4), 683–702. http://doi.org/10.3390/toxins5040683

Ho, V. S. M., Wong, J. H., & Ng, T. B. (2007). A thaumatin-like antifungal protein from the emperor banana. *Peptides*, *28*(4), 760–766. http://doi.org/10.1016/j.peptides.2007.01.005

Hückelhoven, R. (2007). Cell wall-associated mechanisms of disease resistance and susceptibility. *Annual Review of Phytopathology*, *45*, 101–127. http://doi.org/10.1146/annurev.phyto.45.062806.094325

Igor Cesarino, P. A. (2012). An overview of lignin metabolism and its effect on biomass recalcitrance. *Brazilian Journal of Botany*, *35*(4), 303–311. http://doi.org/10.1590/S0100-84042012000400003

Ilmén, M., Thrane, C., & Penttilä, M. (1996). The glucose repressor gene cre1 of Trichoderma: isolation and expression of a full-length and a truncated mutant form. *Molecular & General Genetics: MGG*, *251*(4), 451–460.

Jalal, M. A., Love, S. K., & van der Helm, D. (1986). Siderophore mediated iron(III) uptake in Gliocladium virens. 1. Properties of cis-fusarinine, trans-fusarinine, dimerum acid, and their ferric complexes. *Journal of Inorganic Biochemistry*, *28*(4), 417–430.

Jeffrey G Ellis, P. N. D. (2011). Showdown at the RXLR motif: serious differences of opinion in how effector proteins from filamentous eukaryotic pathogens enter plant cells. PNAS, 108, 14381-14382. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(35), 14381–2. http://doi.org/10.1073/pnas.1111668108

Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., & Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *The EMBO Journal*, *19*(15), 4004–4014. http://doi.org/10.1093/emboj/19.15.4004

Johnson, L. J., Koulman, A., Christensen, M., Lane, G. A., Fraser, K., Forester, N., … Rasmussen, S. (2013). An Extracellular Siderophore Is Required to Maintain the Mutualistic Interaction of Epichloë festucae with Lolium perenne. *PLoS Pathog*, *9*(5), e1003332. http://doi.org/10.1371/journal.ppat.1003332

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323–329. http://doi.org/10.1038/nature05286

Kadonaga, J. T. (2004). Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. *Cell*, *116*(2), 247–257.

Kamoun, S. (2006). A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology*, *44*, 41–60. http://doi.org/10.1146/annurev.phyto.44.070505.143436

Kamoun, S. (2007). Groovy times: filamentous pathogen effectors revealed. *Current Opinion in Plant Biology*, *10*(4), 358–365. http://doi.org/10.1016/j.pbi.2007.04.017

Kang, K., Wang, S., Lai, G., Liu, G., & Xing, M. (2013). Characterization of a novel swollenin from Penicillium oxalicum in facilitating enzymatic saccharification of cellulose. *BMC Biotechnology*, *13*(1), 42. http://doi.org/10.1186/1472-6750-13-42

Kanneganti, V., & Gupta, A. K. (2008). Wall associated kinases from plants — an overview. *Physiology and Molecular Biology of Plants*, *14*(1-2), 109–118. http://doi.org/10.1007/s12298-008-0010-6

Khatabi, B., Molitor, A., Lindermayr, C., Pfiffi, S., Durner, J., von Wettstein, D., … Schäfer, P. (2012). Ethylene Supports Colonization of Plant Roots by the Mutualistic Fungus Piriformospora indica. *PLoS ONE*, *7*(4), e35502. http://doi.org/10.1371/journal.pone.0035502

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., & Salzberg, S. L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology*, *14*(4), R36. http://doi.org/10.1186/gb-2013-14-4-r36

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K., & Yamaguchi, I. (1998). Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. *The Journal of Biological Chemistry*, *273*(3), 1654–1661.

Kleemann, J. (2010, September 8). *Identification and functional characterization of secreted effector proteins of the hemibiotrophic fungus Colletotrichum higginsianum* (text.thesis.doctoral). Universität zu Köln. Retrieved from http://kups.ub.uni-koeln.de/4202/

Klich, M. A. (2007). Aspergillus flavus: the major producer of aflatoxin. *Molecular Plant Pathology*, *8*(6), 713–722. http://doi.org/10.1111/j.1364-3703.2007.00436.x

Koeck, M., Hardham, A. R., & Dodds, P. N. (2011). The role of effectors of biotrophic and hemibiotrophic fungi in infection. *Cellular Microbiology*, *13*(12), 1849–1857. http://doi.org/10.1111/j.1462-5822.2011.01665.x

Kombrink, A., Sánchez-Vallet, A., & Thomma, B. P. H. J. (2011). The role of chitin detection in plant--pathogen interactions. *Microbes and Infection / Institut Pasteur*, *13*(14-15), 1168–1176. http://doi.org/10.1016/j.micinf.2011.07.010

Koo, A. J. K., & Howe, G. A. (2009). The wound hormone jasmonate. *Phytochemistry*, *70*(13-14), 1571–1580. http://doi.org/10.1016/j.phytochem.2009.07.018

Krol, E., Mentzel, T., Chinchilla, D., Boller, T., Felix, G., Kemmerling, B., … Hedrich, R. (2010). Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. *The Journal of Biological Chemistry*, *285*(18), 13471–13479. http://doi.org/10.1074/jbc.M109.097394

Kubicek, C. P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D. A., Druzhinina, I. S., Thon, M., … Grigoriev, I. V. (2011). Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of Trichoderma. *Genome Biology*, *12*(4), R40. http://doi.org/10.1186/gb-2011-12-4-r40

Kubicek, C. P., Starr, T. L., & Glass, N. L. (2014). Plant Cell Wall–Degrading Enzymes and Their Secretion in Plant-Pathogenic Fungi. *Annual Review of Phytopathology*, *52*(1), 427–451. http://doi.org/10.1146/annurev-phyto-102313-045831

Küfner, I., Ottmann, C., Oecking, C., & Nürnberger, T. (2009). Cytolytic toxins as triggers of plant immune response. *Plant Signaling & Behavior*, *4*(10), 977–979.

Kuhn, D. N., Chappell, J., Boudet, A., & Hahlbrock, K. (1984). Induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in cultured plant cells by UV light or fungal elicitor. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(4), 1102–1106.

Kundrotas, P. J., & Alexov, E. (2007). PROTCOM: searchable database of protein complexes enhanced with domain-domain structures. *Nucleic Acids Research*, *35*(Database issue), D575–579. http://doi.org/10.1093/nar/gkl768

Kuo, H.-C., Hui, S., Choi, J., Asiegbu, F. O., Valkonen, J. P. T., & Lee, Y.-H. (2014). Secret lifestyles of Neurospora crassa. *Scientific Reports*, *4*. http://doi.org/10.1038/srep05135

Kurzbaum, E., Kirzhner, F., & Armon, R. (2014). A hydroponic system for growing gnotobiotic vs. sterile plants to study phytoremediation processes. *International Journal of Phytoremediation*, *16*(3), 267–274. http://doi.org/10.1080/15226514.2013.773278

Lacava, P. T., Silva-Stenico, M. E., Araújo, W. L., Simionato, A. V. C., Carrilho, E., Tsai, S. M., & Azevedo, J. L. (2008). Detection of siderophores in endophytic bacteria Methylobacterium spp. associated with Xylella fastidiosa subsp. pauca. *Pesquisa Agropecuária Brasileira*, *43*(4), 521–528. http://doi.org/10.1590/S0100-204X2008000400011

Lah, L., Podobnik, B., Novak, M., Korošec, B., Berne, S., Vogelsang, M., … Komel, R. (2011). The versatility of the fungal cytochrome P450 monooxygenase system is instrumental in xenobiotic detoxification. *Molecular Microbiology*, *81*(5), 1374–1389. http://doi.org/10.1111/j.1365-2958.2011.07772.x

Lalithakumari, D., Mrinalini, C., Chandra, A. B., & Annamalai, P. (1996). Strain improvement by protoplast fusion for enhancement of biocontrol potential integrated with fungicide tolerance in Trichoderma spp. / Stammverbesserung durch Fusion der Protoplasten von Trichoderma-Arten zur Steigerung der Leistungsfähigkeit in der biologischen Bekämpfung integriert mit Fungizidtoleranz. *Zeitschrift Für Pflanzenkrankheiten Und Pflanzenschutz / Journal of Plant Diseases and Protection*, *103*(2), 206–212.

Lanver, D., Mendoza-Mendoza, A., Brachmann, A., & Kahmann, R. (2010). Sho1 and Msb2-related proteins regulate appressorium development in the smut fungus Ustilago maydis. *The Plant Cell*, *22*(6), 2085–2101. http://doi.org/10.1105/tpc.109.073734

Lattanzio, V., Lattanzio, V. M. T., & Cardinali, A. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects., 23–67.

Laugé, R., & De Wit, P. J. (1998). Fungal avirulence genes: structure and possible functions. *Fungal Genetics and Biology: FG & B*, *24*(3), 285–297. http://doi.org/10.1006/fgbi.1998.1076

Le Crom, S., Devaux, F., Marc, P., Zhang, X., Moye-Rowley, W. S., & Jacq, C. (2002). New insights into the pleiotropic drug resistance network from genome-wide characterization of the YRR1 transcription factor regulation system. *Molecular and Cellular Biology*, *22*(8), 2642–2649.

Lee, B.-N., Kroken, S., Chou, D. Y. T., Robbertse, B., Yoder, O. C., & Turgeon, B. G. (2005). Functional Analysis of All Nonribosomal Peptide Synthetases in Cochliobolus heterostrophus Reveals a Factor, NPS6, Involved in Virulence and Resistance to Oxidative Stress. *Eukaryotic Cell*, *4*(3), 545–555. http://doi.org/10.1128/EC.4.3.545-555.2005

Leone, P., Menu-Bouaouiche, L., Peumans, W. J., Payan, F., Barre, A., Roussel, A., … Rougé, P. (2006). Resolution of the structure of the allergenic and antifungal banana fruit thaumatin-like protein at 1.7-A. *Biochimie*, *88*(1), 45–52. http://doi.org/10.1016/j.biochi.2005.07.001

Lichius, A., Bidard, F., Buchholz, F., Crom, S. L., Martin, J., Schackwitz, W., … Kubicek, C. P. (2015). Genome sequencing of the Trichoderma reesei QM9136 mutant identifies a truncation of the transcriptional regulator XYR1 as the cause for its cellulase-negative phenotype. *BMC Genomics*, *16*(1), 326. http://doi.org/10.1186/s12864-015-1526-0

Lindstrom, J. T., Sun, S., & Belanger, F. C. (1993). A Novel Fungal Protease Expressed in Endophytic Infection of Poa Species. *Plant Physiology*, *102*(2), 645–650.

Lionetti, V., Cervone, F., & Bellincampi, D. (2012). Methyl esterification of pectin plays a role during plant-pathogen interactions and affects plant resistance to diseases. *Journal of Plant Physiology*, *169*(16), 1623–1630. http://doi.org/10.1016/j.jplph.2012.05.006

Li, Y., Zhang, Q., Zhang, J., Wu, L., Qi, Y., & Zhou, J.-M. (2010). Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiology*, *152*(4), 2222–2231. http://doi.org/10.1104/pp.109.151803

Lorito, M., Hayes, C. K., Di Pietro, A., & Harman, G. E. (1993). Biolistic transformation of Trichoderma harzianum and Gliocladium virens using plasmid and genomic DNA. *Current Genetics*, *24*(4), 349–356.

Lo, S.-F., Yang, S.-Y., Chen, K.-T., Hsing, Y.-I., Zeevaart, J. A. D., Chen, L.-J., & Yu, S.-M. (2008). A Novel Class of Gibberellin 2-Oxidases Control Semidwarfism, Tillering, and Root Development in Rice. *The Plant Cell Online*, *20*(10), 2603–2618. http://doi.org/10.1105/tpc.108.060913

Lu, H. (2009). Dissection of salicylic acid-mediated defense signaling networks. *Plant Signaling & Behavior*, *4*(8), 713–717.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., & Ton, J. (2011). Callose deposition: a multifaceted plant defense response. *Molecular Plant-Microbe Interactions: MPMI*, *24*(2), 183–193. http://doi.org/10.1094/MPMI-07-10-0149

Lund, S. T., Stall, R. E., & Klee, H. J. (1998). Ethylene Regulates the Susceptible Response to Pathogen Infection in Tomato. *The Plant Cell*, *10*(3), 371–382. http://doi.org/10.1105/tpc.10.3.371

Lysøe, E., Seong, K.-Y., & Kistler, H. C. (2011). The transcriptome of Fusarium graminearum during the infection of wheat. *Molecular Plant-Microbe Interactions: MPMI*, *24*(9), 995–1000. http://doi.org/10.1094/MPMI-02-11-0038

Mackey, D., Holt, B. F., Wiig, A., & Dangl, J. L. (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. *Cell*, *108*(6), 743–754.

MacPherson, S., Larochelle, M., & Turcotte, B. (2006). A Fungal Family of Transcriptional Regulators: the Zinc Cluster Proteins. *Microbiology and Molecular Biology Reviews*, *70*(3), 583–604. http://doi.org/10.1128/MMBR.00015-06

Malinovsky, F. G., Fangel, J. U., & Willats, W. G. T. (2014). The role of the cell wall in plant immunity. *Frontiers in Plant Science*, *5*. http://doi.org/10.3389/fpls.2014.00178

Mańka, M. (1980). Auxin and gibberellin-like substances synthesis by Fusarium isolates pathogenic to corn seedlings. *Acta Microbiologica Polonica*, *29*(4), 365–374.

Marina Tucci, M. R. (2011). The beneficial effect of Trichoderma spp. on tomato is modulated by the plant genotype. *Molecular Plant Pathology*, *12*(4), 341–54. http://doi.org/10.1111/j.1364-3703.2010.00674.x

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K. E., … Rudd, J. J. (2011). Analysis of two in planta expressed LysM effector homologs from the fungus Mycosphaerella graminicola reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology*, *156*(2), 756–769. http://doi.org/10.1104/pp.111.176347

Martinez, D., Berka, R. M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S. E., … Brettin, T. S. (2008). Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). *Nature Biotechnology*, *26*(5), 553–560. http://doi.org/10.1038/nbt1403

Martin, F., Aerts, A., Ahrén, D., Brun, A., Danchin, E. G. J., Duchaussoy, F., … Grigoriev, I. V. (2008). The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. *Nature*, *452*(7183), 88–92. http://doi.org/10.1038/nature06556

Martin, G. B., Bogdanove, A. J., & Sessa, G. (2003). Understanding the Functions of Plant Disease Resistance Proteins. *Annual Review of Plant Biology*, *54*(1), 23–61. http://doi.org/10.1146/annurev.arplant.54.031902.135035

Masloff, S., Jacobsen, S., Pöggeler, S., & Kück, U. (2002). Functional analysis of the C6 zinc finger gene pro1 involved in fungal sexual development. *Fungal Genetics and Biology: FG & B*, *36*(2), 107–116. http://doi.org/10.1016/S1087-1845(02)00010-5

Masloff, S., Pöggeler, S., & Kück, U. (1999). The pro1(+) gene from Sordaria macrospora encodes a C6 zinc finger transcription factor required for fruiting body development. *Genetics*, *152*(1), 191–199.

Mayer, A., di Iulio, J., Maleri, S., Eser, U., Vierstra, J., Reynolds, A., … Churchman, L. S. (2015). Native elongating transcript sequencing reveals human transcriptional activity at nucleotide resolution. *Cell*, *161*(3), 541–554. http://doi.org/10.1016/j.cell.2015.03.010

McHale, L., Tan, X., Koehl, P., & Michelmore, R. W. (2006). Plant NBS-LRR proteins: adaptable guards. *Genome Biology*, *7*(4), 212. http://doi.org/10.1186/gb-2006-7-4-212

Mehrdad Azin, R. M. (2007). Production of xylanase by Trichoderma longibrachiatum on a mixture of wheat bran and wheat straw: Optimization of culture condition by Taguchi method. *Enzyme and Microbial Technology*, *40*(4), 801–805. http://doi.org/10.1016/j.enzmictec.2006.06.013

Mendoza-Mendoza, A., Pozo, M. J., Grzegorski, D., Martínez, P., García, J. M., Olmedo-Monfil, V., … Herrera-Estrella, A. (2003). Enhanced biocontrol activity of Trichoderma through inactivation of a mitogen-activated protein kinase. *Proceedings of the National Academy of*

Sciences of the United States of America, *100*(26), 15965–15970. http://doi.org/10.1073/pnas.2136716100

Mentlak, T. A., Kombrink, A., Shinya, T., Ryder, L. S., Otomo, I., Saitoh, H., … Talbot, N. J. (2012). Effector-mediated suppression of chitin-triggered immunity by magnaporthe oryzae is necessary for rice blast disease. *The Plant Cell*, *24*(1), 322–335. http://doi.org/10.1105/tpc.111.092957

Mesarich, C. H., Bowen, J. K., Hamiaux, C., & Templeton, M. D. (2015). Repeat-containing protein effectors of plant-associated organisms. *Frontiers in Plant Science*, *6*. http://doi.org/10.3389/fpls.2015.00872

Moghaddam, M. R. B., & Ende, W. V. den. (2012). Sugars and plant innate immunity. *Journal of Experimental Botany*, ers129. http://doi.org/10.1093/jxb/ers129

Mohnen, D. (2008). Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, *11*(3), 266–277. http://doi.org/10.1016/j.pbi.2008.03.006

Morán-Diez, M. E., Trushina, N., Lamdan, N. L., Rosenfelder, L., Mukherjee, P. K., Kenerley, C. M., & Horwitz, B. A. (2015). Host-specific transcriptomic pattern of Trichoderma virens during interaction with maize or tomato roots. *BMC Genomics*, *16*(1), 8. http://doi.org/10.1186/s12864-014-1208-3

Mosavi, L. K., Cammett, T. J., Desrosiers, D. C., & Peng, Z. (2004). The ankyrin repeat as molecular architecture for protein recognition. *Protein Science : A Publication of the Protein Society*, *13*(6), 1435–1448. http://doi.org/10.1110/ps.03554604

Motteram, J., Küfner, I., Deller, S., Brunner, F., Hammond-Kosack, K. E., Nürnberger, T., & Rudd, J. J. (2009). Molecular characterization and functional analysis of MgNLP, the sole NPP1 domain-containing protein, from the fungal wheat leaf pathogen Mycosphaerella

graminicola. *Molecular Plant-Microbe Interactions: MPMI*, *22*(7), 790–799. http://doi.org/10.1094/MPMI-22-7-0790

Moy, M., Li, H. M., Sullivan, R., White, J. F., & Belanger, F. C. (2002). Endophytic Fungal β-1,6-Glucanase Expression in the Infected Host Grass. *Plant Physiology*, *130*(3), 1298–1308. http://doi.org/10.1104/pp.010108

MSD Animal Health New Zealand - Home. (n.d.). Retrieved July 22, 2015, from http://www.msd-animal-health.co.nz/

Mukherjee, P. K., Buensanteai, N., Moran-Diez, M. E., Druzhinina, I. S., & Kenerley, C. M. (2012). Functional analysis of non-ribosomal peptide synthetases (NRPSs) in Trichoderma virens reveals a polyketide synthase (PKS)/NRPS hybrid enzyme involved in the induced systemic resistance response in maize. *Microbiology*, *158*(1), 155–165. http://doi.org/10.1099/mic.0.052159-0

Mukherjee, P. K., Horwitz, B. A., & Kenerley, C. M. (2012). Secondary metabolism in Trichoderma – a genomic perspective. *Microbiology*, *158*(1), 35–45. http://doi.org/10.1099/mic.0.053629-0

Mukherjee, P. K., Horwitz, B. A., Singh, U. S., Mukherjee, M., & Schmoll, M. (2013). *Trichoderma: Biology and Applications*. CABI.

Müller, O., Schreier, P. H., & Uhrig, J. F. (2008). Identification and characterization of secreted and pathogenesis-related proteins in Ustilago maydis. *Molecular Genetics and Genomics: MGG*, *279*(1), 27–39. http://doi.org/10.1007/s00438-007-0291-4

Murphy, B., Hodkinson, T., & Doohan, F. (2013). Mechanisms of beneficial colonisation of barley by fungal root endophytes, *120*(2013), 35–44.

Muthamilarasan, M., & Prasad, M. (2013). Plant innate immunity: an updated insight into defense mechanism. *Journal of Biosciences*, *38*(2), 433–449.

Natesan Balasubramanian, D. L. (2008). Characteristics of protoplast inter-, intra-fusant and regeneration of antagonistic fungi Trichoderma harzianum and Trichoderma viride. Afr J Biotechnol 7:3235-3243. *African Journal of Biotechnology*, *7*(18), 3235–3243.

Nautiyal, C. S., & Dion, P. (2008). *Molecular Mechanisms of Plant and Microbe Coexistence*. Springer Science & Business Media.

Naznin, H. A., Kiyohara, D., Kimura, M., Miyazawa, M., Shimizu, M., & Hyakumachi, M. (2014). Systemic Resistance Induced by Volatile Organic Compounds Emitted by Plant Growth-Promoting Fungi in Arabidopsis thaliana. *PLoS ONE*, *9*(1), e86882. http://doi.org/10.1371/journal.pone.0086882

Nicolas Denancé, P. R. (2012). Arabidopsis wat1 (walls are thin1)-mediated resistance to the bacterial vascular pathogen, Ralstonia solanacearum, is accompanied by cross-regulation of salicylic acid and tryptophan metabolism. *The Plant Journal : For Cell and Molecular Biology*. http://doi.org/10.1111/tpj.12027

N. SACHIN, B. L. M. (2013). Do endophytic fungi possess pathway genes for plant secondary metabolites? *Current Science*, *104*(2).

O'Brien, J. A., Daudi, A., Finch, P., Butt, V. S., Whitelegge, J. P., Souda, P., … Bolwell, G. P. (2012). A peroxidase-dependent apoplastic oxidative burst in cultured Arabidopsis cells functions in MAMP-elicited defense. *Plant Physiology*, *158*(4), 2013–2027. http://doi.org/10.1104/pp.111.190140

Ochoa-Villarreal, M., Aispuro-Hernández, E., & Martínez-Téllez, I. V.-A. and M. Á. (2012). Plant Cell Wall Polymers: Function, Structure and Biological Activity of Their Derivatives. http://doi.org/10.5772/46094

O'Connell, R. J., Thon, M. R., Hacquard, S., Amyotte, S. G., Kleemann, J., Torres, M. F., … Vaillancourt, L. J. (2012). Lifestyle transitions in plant pathogenic Colletotrichum fungi

deciphered by genome and transcriptome analyses. *Nature Genetics*, *44*(9), 1060–1065. http://doi.org/10.1038/ng.2372

Oh, Y., Donofrio, N., Pan, H., Coughlan, S., Brown, D. E., Meng, S., … Dean, R. A. (2008). Transcriptome analysis reveals new insight into appressorium formation and function in the rice blast fungus Magnaporthe oryzae. *Genome Biology*, *9*(5), R85. http://doi.org/10.1186/gb-2008-9-5-r85

Olivain, C., Humbert, C., Nahalkova, J., Fatehi, J., L'Haridon, F., & Alabouvette, C. (2006). Colonization of Tomato Root by Pathogenic and Nonpathogenic Fusarium oxysporum Strains Inoculated Together and Separately into the Soil. *Applied and Environmental Microbiology*, *72*(2), 1523–1531. http://doi.org/10.1128/AEM.72.2.1523-1531.2006

Oliva, R., Win, J., Raffaele, S., Boutemy, L., Bozkurt, T. O., Chaparro-Garcia, A., … Kamoun, S. (2010). Recent developments in effector biology of filamentous plant pathogens. *Cellular Microbiology*, *12*(7), 1015. http://doi.org/10.1111/j.1462-5822.2010.01484.x

Omann, M., & Zeilinger, S. (2010). How a Mycoparasite Employs G-Protein Signaling: Using the Example of Trichoderma. *Journal of Signal Transduction*, *2010*. http://doi.org/10.1155/2010/123126

Osborn, H. M. I., Lochey, F., Mosley, L., & Read, D. (1999). Analysis of polysaccharides and monosaccharides in the root mucilage of maize (Zea mays L.) by gas chromatography. *Journal of Chromatography, A*, *831*(2), 267–276. http://doi.org/10.1016/S0021-9673(98)00935-2

Ottmann, C., Luberacki, B., Küfner, I., Koch, W., Brunner, F., Weyand, M., … Oecking, C. (2009). A common toxin fold mediates microbial attack and plant defense. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(25), 10359–10364. http://doi.org/10.1073/pnas.0902362106

Pandey, S. P., & Somssich, I. E. (2009). The Role of WRKY Transcription Factors in Plant Immunity. *Plant Physiology*, *150*(4), 1648–1655. http://doi.org/10.1104/pp.109.138990

Panina, E. M., Mironov, A. A., & Gelfand, M. S. (2003). Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(17), 9912–9917. http://doi.org/10.1073/pnas.1733691100

Peij, N. N. M. E. van, Gielkens, M. M. C., Vries, R. P. de, Visser, J., & Graaff, L. H. de. (n.d.). The Transcriptional Activator XlnR Regulates Both Xylanolytic and Endoglucanase Gene Expression inAspergillus niger. Retrieved July 29, 2014, from http://aem.asm.org

Pérez-Nadales, E., & Di Pietro, A. (2011). The membrane mucin Msb2 regulates invasive growth and plant infection in Fusarium oxysporum. *The Plant Cell*, *23*(3), 1171–1185. http://doi.org/10.1105/tpc.110.075093

Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods*, *8*(10), 785–786. http://doi.org/10.1038/nmeth.1701

Peterson, R., & Nevalainen, H. (2012). Trichoderma reesei RUT-C30--thirty years of strain improvement. *Microbiology (Reading, England)*, *158*(Pt 1), 58–68. http://doi.org/10.1099/mic.0.054031-0

Petre, B., & Kamoun, S. (2014). How Do Filamentous Pathogens Deliver Effector Proteins into Plant Cells? *PLoS Biology*, *12*(2). http://doi.org/10.1371/journal.pbio.1001801

Presti, L. L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., … Kahmann, R. (2015). Fungal Effectors and Plant Susceptibility. *Annual Review of Plant Biology*, *66*(1), 513–545. http://doi.org/10.1146/annurev-arplant-043014-114623

Pritchard, L., & Birch, P. (2011). A systems biology perspective on plant-microbe interactions: biochemical and structural targets of pathogen effectors. *Plant Science: An International Journal of Experimental Plant Biology*, *180*(4), 584–603. http://doi.org/10.1016/j.plantsci.2010.12.008

Pritchard, L., & Birch, P. R. J. (2014). The zigzag model of plant–microbe interactions: is it time to move on? *Molecular Plant Pathology*, *15*(9), 865–870. http://doi.org/10.1111/mpp.12210

Rafiqi, M., Ellis, J. G., Ludowici, V. A., Hardham, A. R., & Dodds, P. N. (2012). Challenges and progress towards understanding the role of effectors in plant-fungal interactions. *Current Opinion in Plant Biology*, *15*(4), 477–482. http://doi.org/10.1016/j.pbi.2012.05.003

Rafiqi, M., Jelonek, L., Akum, N. F., Zhang, F., & Kogel, K.-H. (2013). Effector candidates in the secretome of Piriformospora indica, a ubiquitous plant-associated fungus. *Frontiers in Plant Science*, *4*. http://doi.org/10.3389/fpls.2013.00228

Rasmussen, S., Parsons, A. J., Popay, A., Xue, H., & Newman, J. A. (2008). Plant-endophyte-herbivore interactions. *Plant Signaling & Behavior*, *3*(11), 974–977.

Reddy, P. V., Lam, C. K., & Belanger, F. C. (1996). Mutualistic fungal endophytes express a proteinase that is homologous to proteases suspected to be important in fungal pathogenicity. *Plant Physiology*, *111*(4), 1209–1218.

Richard S Quilliam, D. L. J. (2012). Evidence for host-specificity of culturable fungal root endophytes from the carnivorous plant Pinguicula vulgaris (Common Butterwort). *Mycological Progress*, *11*(2).

Rich, M. K., Schorderet, M., & Reinhardt, D. (2014). The role of the cell wall compartment in mutualistic symbioses of plants. *Frontiers in Plant Science*, *5*, 238. http://doi.org/10.3389/fpls.2014.00238

Robinson, M. D., McCarthy, D. J., & Robinson, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140. http://doi.org/10.1093/bioinformatics/btp616

Rocha-Ramírez, V., Omero, C., Chet, I., Horwitz, B. A., & Herrera-Estrella, A. (2002). Trichoderma atroviride G-Protein α-Subunit Gene tga1 Is Involved in Mycoparasitic Coiling and Conidiation. *Eukaryotic Cell*, *1*(4), 594–605. http://doi.org/10.1128/EC.1.4.594-605.2002

Rodríguez, A., Shimada, T., Cervera, M., Alquézar, B., Gadea, J., Gómez-Cadenas, A., … Peña, L. (2014). Terpene Down-Regulation Triggers Defense Responses in Transgenic Orange Leading to Resistance against Fungal Pathogens. *Plant Physiology*, *164*(1), 321–339. http://doi.org/10.1104/pp.113.224279

Rodriguez, R. J., White, J. F., Arnold, A. E., & Redman, R. S. (2009). Fungal endophytes: diversity and functional roles. *The New Phytologist*, *182*(2), 314–330. http://doi.org/10.1111/j.1469-8137.2009.02773.x

Ron, M., & Avni, A. (2004). The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *The Plant Cell*, *16*(6), 1604–1615. http://doi.org/10.1105/tpc.022475

Rosso, M.-N., Vieira, P., de Almeida-Engler, J., & Castagnone-Sereno, P. (2011). Proteins secreted by root-knot nematodes accumulate in the extracellular compartment during root infection. *Plant Signaling & Behavior*, *6*(8), 1232–1234. http://doi.org/10.4161/psb.6.8.16290

Rotblat, B., Enshell-Seijffers, D., Gershoni, J. M., Schuster, S., & Avni, A. (2002). Identification of an essential component of the elicitation active site of the EIX protein elicitor. *The Plant Journal: For Cell and Molecular Biology*, *32*(6), 1049–1055.

Ruiz-Díez, B. (2002). Strategies for the transformation of filamentous fungi. *Journal of Applied Microbiology*, *92*(2), 189–195. http://doi.org/10.1046/j.1365-2672.2002.01516.x

Rushton, P. J., Somssich, I. E., Ringler, P., & Shen, Q. J. (2010). WRKY transcription factors. *Trends in Plant Science*, *15*(5), 247–258. http://doi.org/10.1016/j.tplants.2010.02.006

Salas-Marina, M. A., Isordia-Jasso, M. I., Islas-Osuna, M. A., Delgado-Sánchez, P., Jiménez-Bremont, J. F., Rodríguez-Kessler, M., … Casas-Flores, S. (2015). The Epl1 and Sm1 proteins from Trichoderma atroviride and Trichoderma virens differentially modulate systemic disease resistance against different life style pathogens in Solanum lycopersicum. *Frontiers in Plant Science*, *6*. http://doi.org/10.3389/fpls.2015.00077

Saloheimo, M., Paloheimo, M., Hakola, S., Pere, J., Swanson, B., Nyyssönen, E., … Penttilä, M. (2002). Swollenin, a Trichoderma reesei protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. *European Journal of Biochemistry / FEBS*, *269*(17), 4202–4211.

Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*. CSHL Press.

Sánchez-Vallet, A., Mesters, J. R., & Thomma, B. P. H. J. (2015). The battle for chitin recognition in plant-microbe interactions. *FEMS Microbiology Reviews*, *39*(2), 171–183. http://doi.org/10.1093/femsre/fuu003

Sargent, F., Berks, B. C., & Palmer, T. (2006). Pathfinders and trailblazers: a prokaryotic targeting system for transport of folded proteins. *FEMS Microbiology Letters*, *254*(2), 198–207. http://doi.org/10.1111/j.1574-6968.2005.00049.x

Saunders, D. G. O., Win, J., Cano, L. M., Szabo, L. J., Kamoun, S., & Raffaele, S. (2012). Using hierarchical clustering of secreted protein families to classify and rank candidate effectors of rust fungi. *PloS One*, *7*(1), e29847. http://doi.org/10.1371/journal.pone.0029847

Schäfer, P., Pfiffi, S., Voll, L. M., Zajic, D., Chandler, P. M., Waller, F., … Kogel, K.-H. (2009). Manipulation of plant innate immunity and gibberellin as factor of compatibility in the

mutualistic association of barley roots with Piriformospora indica. *The Plant Journal: For Cell and Molecular Biology*, *59*(3), 461–474. http://doi.org/10.1111/j.1365-313X.2009.03887.x

Schardl, C. L., Grossman, R. B., Nagabhyru, P., Faulkner, J. R., & Mallik, U. P. (2007). Loline alkaloids: Currencies of mutualism. *Phytochemistry*, *68*(7), 980–996. http://doi.org/10.1016/j.phytochem.2007.01.010

Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Annual Review of Plant Biology*, *61*(1), 263–289. http://doi.org/10.1146/annurev-arplant-042809-112315

Scholze, H., & Boch, J. (2010). TAL effector-DNA specificity. *Virulence*, *1*(5), 428–432. http://doi.org/10.4161/viru.1.5.12863

Schulz, B., Wanke, U., Draeger, S., & Aust, H.-J. (1993). Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological Research*, *97*(12), 1447–1450. http://doi.org/10.1016/S0953-7562(09)80215-3

Schulze-Lefert, P., & Robatzek, S. (2006). Plant Pathogens Trick Guard Cells into Opening the Gates. *Cell*, *126*(5), 831–834. http://doi.org/10.1016/j.cell.2006.08.020

Seiboth, B., & Seidl-Seiboth, C. I. and V. (2011). Trichoderma reesei: A Fungal Enzyme Producer for Cellulosic Biofuels. http://doi.org/10.5772/16848

Seidl-Seiboth, V., Gruber, S., Sezerman, U., Schwecke, T., Albayrak, A., Neuhof, T., … Kubicek, C. P. (2011). Novel hydrophobins from Trichoderma define a new hydrophobin subclass: protein properties, evolution, regulation and processing. *Journal of Molecular Evolution*, *72*(4), 339–351. http://doi.org/10.1007/s00239-011-9438-3

Seidl-Seiboth, V., Zach, S., Frischmann, A., Spadiut, O., Dietzsch, C., Herwig, C., … Kubicek, C. P. (2013). Spore germination of Trichoderma atroviride is inhibited by its LysM protein TAL6. *The FEBS Journal*, *280*(5), 1226–1236. http://doi.org/10.1111/febs.12113

Semple, C. A. M., RIKEN GER Group, & GSL Members. (2003). The comparative proteomics of ubiquitination in mouse. *Genome Research*, *13*(6B), 1389–1394. http://doi.org/10.1101/gr.980303

Senchou, V., Weide, R., Carrasco, A., Bouyssou, H., Pont-Lezica, R., Govers, F., & Canut, H. (2004). High affinity recognition of a Phytophthora protein by Arabidopsis via an RGD motif. *Cellular and Molecular Life Sciences: CMLS*, *61*(4), 502–509. http://doi.org/10.1007/s00018-003-3394-z

Shabab, M., Shindo, T., Gu, C., Kaschani, F., Pansuriya, T., Chintha, R., … van der Hoorn, R. A. L. (2008). Fungal effector protein AVR2 targets diversifying defense-related cys proteases of tomato. *The Plant Cell*, *20*(4), 1169–1183. http://doi.org/10.1105/tpc.107.056325

Shah, J. (2009). Plants under attack: systemic signals in defence. *Current Opinion in Plant Biology*, *12*(4), 459–464. http://doi.org/10.1016/j.pbi.2009.05.011

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N. G., Palmer, A. E., & Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nature Biotechnology*, *22*(12), 1567–1572. http://doi.org/10.1038/nbt1037

Shishido, M., Breuil, C., & Chanway, C. P. (1999). Endophytic colonization of spruce by plant growth-promoting rhizobacteria. *FEMS Microbiology Ecology*, *29*(2), 191–196. http://doi.org/10.1111/j.1574-6941.1999.tb00610.x

Shoresh, M., & Harman, G. E. (2008). The molecular basis of shoot responses of maize seedlings to Trichoderma harzianum T22 inoculation of the root: a proteomic approach. *Plant Physiology*, *147*(4), 2147–2163. http://doi.org/10.1104/pp.108.123810

Shoresh, M., Harman, G. E., & Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annual Review of Phytopathology*, *48*, 21–43. http://doi.org/10.1146/annurev-phyto-073009-114450

Shoresh, M., Yedidia, I., & Chet, I. (2005). Involvement of Jasmonic Acid/Ethylene Signaling Pathway in the Systemic Resistance Induced in Cucumber by Trichoderma asperellum T203. *Phytopathology*, *95*(1), 76–84. http://doi.org/10.1094/PHYTO-95-0076

Sinha, D., Gupta, M. K., Patel, H. K., Ranjan, A., & Sonti, R. V. (2013). Cell Wall Degrading Enzyme Induced Rice Innate Immune Responses Are Suppressed by the Type 3 Secretion System Effectors XopN, XopQ, XopX and XopZ of Xanthomonas oryzae pv. oryzae. *PLoS ONE*, *8*(9), e75867. http://doi.org/10.1371/journal.pone.0075867

Smith, L. G. (2001). Plant cell division: building walls in the right places. *Nature Reviews Molecular Cell Biology*, *2*(1), 33–39. http://doi.org/10.1038/35048050

Sobowale A. A., B. O. O. (2011). Abilities of Trichoderma species to persist within Maize (Zea mays) stem long after inoculation. *British Microbiology Research Journal*, *1*(4), 95–103.

Sobowale, A. A., Cardwell, K. F., Odebode, A. C., Bandyopadhyay, R., & Jonathan, S. G. (2007). Persistence of Trichoderma species within maize stem against Fusarium verticillioides. *Archives of Phytopathology and Plant Protection*, *40*(3), 215–231. http://doi.org/10.1080/03235400500424596

Sperschneider, J., Dodds, P. N., Gardiner, D. M., Manners, J. M., Singh, K. B., & Taylor, J. M. (2015). Advances and Challenges in Computational Prediction of Effectors from Plant Pathogenic Fungi. *PLoS Pathog*, *11*(5), e1004806. http://doi.org/10.1371/journal.ppat.1004806

Staples, R. C. (2001). Nutrients for a rust fungus: the role of haustoria. *Trends in Plant Science*, *6*(11), 496–498. http://doi.org/10.1016/S1360-1385(01)02126-4

Stassen, J. H. M., & Van den Ackerveken, G. (2011). How do oomycete effectors interfere with plant life? *Current Opinion in Plant Biology*, *14*(4), 407–414. http://doi.org/10.1016/j.pbi.2011.05.002

Stergiopoulos, I., & de Wit, P. J. G. M. (2009). Fungal effector proteins. *Annual Review of Phytopathology*, *47*, 233–263. http://doi.org/10.1146/annurev.phyto.112408.132637

Stergiopoulos, I., van den Burg, H. A., Okmen, B., Beenen, H. G., van Liere, S., Kema, G. H. J., & de Wit, P. J. G. M. (2010). Tomato Cf resistance proteins mediate recognition of cognate homologous effectors from fungi pathogenic on dicots and monocots. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(16), 7610–7615. http://doi.org/10.1073/pnas.1002910107

Stricker, A. R., Grosstessner-Hain, K., Wurleitner, E., & Mach, R. L. (2006). Xyr1 (Xylanase Regulator 1) Regulates both the Hydrolytic Enzyme System and d-Xylose Metabolism in Hypocrea jecorina. *Eukaryotic Cell*, *5*(12), 2128–2137. http://doi.org/10.1128/EC.00211-06

Stuart, L. M., Paquette, N., & Boyer, L. (2013). Effector-triggered versus pattern-triggered immunity: how animals sense virulent pathogens. *Nature Reviews. Immunology*, *13*(3), 199–206. http://doi.org/10.1038/nri3398

Sundram, S. (2014). First report: Isolation of endophytic trichoderma from oil palm (Elaeis guineensis Jacq.) and their in vitro antagonistic assessment on Ganoderma boninense. *Journal of Oil Palm Research*, *25*(253), 368–372.

Sun, T. (2010). Gibberellin-GID1-DELLA: A Pivotal Regulatory Module for Plant Growth and Development. *Plant Physiology*, *154*(2), 567–570. http://doi.org/10.1104/pp.110.161554

Szabo, L. J., & Bushnell, W. R. (2001). Hidden robbers: The role of fungal haustoria in parasitism of plants. *Proceedings of the National Academy of Sciences*, *98*(14), 7654–7655. http://doi.org/10.1073/pnas.151262398

Takahashi, H., Matsumura, H., Kawai-Yamada, M., & Uchimiya, H. (2008). The cell death factor, cell wall elicitor of rice blast fungus (Magnaporthe grisea) causes metabolic alterations including GABA shunt in rice cultured cells. *Plant Signaling & Behavior*, *3*(11), 945–953.

Takashima, S., Iikura, H., Nakamura, A., Masaki, H., & Uozumi, T. (1996). Analysis of Cre1 binding sites in the Trichoderma reesei cbh1 upstream region. *FEMS Microbiology Letters*, *145*(3), 361–366.

Talbot, N. J. (2010). Living the Sweet Life: How Does a Plant Pathogenic Fungus Acquire Sugar from Plants? *PLoS Biol*, *8*(2), e1000308. http://doi.org/10.1371/journal.pbio.1000308

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, mst197. http://doi.org/10.1093/molbev/mst197

Tauzin, A. S., & Giardina, T. (2014). Sucrose and invertases, a part of the plant defense response to the biotic stresses. *Frontiers in Plant Science*, *5*. http://doi.org/10.3389/fpls.2014.00293

Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., … Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal: For Cell and Molecular Biology*, *37*(6), 914–939.

Thomas, C. M., Jones, D. A., Parniske, M., Harrison, K., Balint-Kurti, P. J., Hatzixanthis, K., & Jones, J. D. (1997). Characterization of the tomato Cf-4 gene for resistance to Cladosporium fulvum identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. *The Plant Cell*, *9*(12), 2209–2224. http://doi.org/10.1105/tpc.9.12.2209

Thonart, P., Ongena, M., & Henry, G. (2012). PAMPs, MAMPs, DAMPs and others: an update on the diversity of plant immunity elicitors. *Base*. Retrieved from http://popups.ulg.ac.be/1780-4507/index.php?id=8709

Tian, D., Traw, M. B., Chen, J. Q., Kreitman, M., & Bergelson, J. (2003). Fitness costs of R-gene-mediated resistance in Arabidopsis thaliana. *Nature*, *423*(6935), 74–77. http://doi.org/10.1038/nature01588

Tobe, T., Beatson, S. A., Taniguchi, H., Abe, H., Bailey, C. M., Fivian, A., … Pallen, M. J. (2006). An extensive repertoire of type III secretion effectors in Escherichia coli O157 and the role of lambdoid phages in their dissemination. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(40), 14941–14946. http://doi.org/10.1073/pnas.0604891103

Tobimatsu, Y., Wagner, A., Donaldson, L., Mitra, P., Niculaes, C., Dima, O., … Ralph, J. (2013). Visualization of plant cell wall lignification using fluorescence-tagged monolignols. *The Plant Journal*, *76*(3), 357–366. http://doi.org/10.1111/tpj.12299

Tognetti, J. A., Pontis, H. G., & Martínez-Noël, G. M. A. (2013). Sucrose signaling in plants: a world yet to be explored. *Plant Signaling & Behavior*, *8*(3), e23316. http://doi.org/10.4161/psb.23316

Traoré, O., Groleau-Renaud, V., Plantureux, S., Tubeileh, A., & Boeuf-Tremblay, V. (2000). Effect of root mucilage and modelled root exudates on soil structure. *European Journal of Soil Science*, *51*(4), 575–581. http://doi.org/10.1111/j.1365-2389.2000.00348.x

Traven, A., Jelicic, B., & Sopta, M. (2006). Yeast Gal4: a transcriptional paradigm revisited. *EMBO Reports*, *7*(5), 496–499. http://doi.org/10.1038/sj.embor.7400679

Troncoso, C., González, X., Bömke, C., Tudzynski, B., Gong, F., Hedden, P., & Rojas, M. C. (2010). Gibberellin biosynthesis and gibberellin oxidase activities in Fusarium sacchari, Fusarium konzum and Fusarium subglutinans strains. *Phytochemistry*, *71*(11-12), 1322–1331. http://doi.org/10.1016/j.phytochem.2010.05.006

Tseng, T.-T., Tyler, B. M., & Setubal, J. C. (2009). Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. *BMC Microbiology*, *9*(Suppl 1), S2. http://doi.org/10.1186/1471-2180-9-S1-S2

Tyler, B. M., Kale, S. D., Wang, Q., Tao, K., Clark, H. R., Drews, K., … Lawrence, C. B. (2013). Microbe-independent entry of oomycete RxLR effectors and fungal RxLR-like effectors into plant and animal cells is specific and reproducible. *Molecular Plant-Microbe Interactions: MPMI*, *26*(6), 611–616. http://doi.org/10.1094/MPMI-02-13-0051-IA

Tzin, V., & Galili, G. (2010). New Insights into the Shikimate and Aromatic Amino Acids Biosynthesis Pathways in Plants. *Molecular Plant*, *3*(6), 956–972. http://doi.org/10.1093/mp/ssq048

Usadel, B., Poree, F., Nagel, A., Lohse, M., Czedik-Eysenberg, A., & Stitt, M. (2009). A guide to using MapMan to visualize and compare Omics data in plants: a case study in the crop species, Maize. *Plant, Cell & Environment*, *32*(9), 1211–1229. http://doi.org/10.1111/j.1365-3040.2009.01978.x

Vadassery, J., Ranf, S., Drzewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., … Oelmüller, R. (2009). A cell wall extract from the endophytic fungus Piriformospora indica promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. *The Plant Journal: For Cell and Molecular Biology*, *59*(2), 193–206. http://doi.org/10.1111/j.1365-313X.2009.03867.x

Valkonen, M., Kalkman, E. R., Saloheimo, M., Penttilä, M., Read, N. D., & Duncan, R. R. (2007). Spatially segregated SNARE protein interactions in living fungal cells. *The Journal of Biological Chemistry*, *282*(31), 22775–22785. http://doi.org/10.1074/jbc.M700916200

Van den Ackerveken, G. F., Van Kan, J. A., & De Wit, P. J. (1992). Molecular analysis of the avirulence gene avr9 of the fungal tomato pathogen Cladosporium fulvum fully supports the gene-for-gene hypothesis. *The Plant Journal: For Cell and Molecular Biology*, *2*(3), 359–366.

Van Engelenburg, S. B., & Palmer, A. E. (2010). Imaging type-III secretion reveals dynamics and spatial segregation of Salmonella effectors. *Nature Methods*, *7*(4), 325–330. http://doi.org/10.1038/nmeth.1437

van Loon, L. C., Geraats, B. P. J., & Linthorst, H. J. M. (2006). Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science*, *11*(4), 184–191. http://doi.org/10.1016/j.tplants.2006.02.005

van Peij, N. N. M. E., Gielkens, M. M. C., de Vries, R. P., Visser, J., & de Graaff, L. H. (1998). The Transcriptional Activator XlnR Regulates Both Xylanolytic and Endoglucanase Gene Expression in Aspergillus niger. *Applied and Environmental Microbiology*, *64*(10), 3615–3619.

Van Peij, N. N. M. E., Visser, J., & De Graaff, L. H. (1998). Isolation and analysis of xlnR, encoding a transcriptional activator co-ordinating xylanolytic expression in Aspergillus niger. *Molecular Microbiology*, *27*(1), 131–142. http://doi.org/10.1046/j.1365-2958.1998.00666.x

Van Roey, K., Uyar, B., Weatheritt, R. J., Dinkel, H., Seiler, M., Budd, A., … Davey, N. E. (2014). Short Linear Motifs: Ubiquitous and Functionally Diverse Protein Interaction Modules Directing Cell Regulation. *Chemical Reviews*, *114*(13), 6733–6778. http://doi.org/10.1021/cr400585q

Van Wees, S. C. M., Van der Ent, S., & Pieterse, C. M. J. (2008). Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology*, *11*(4), 443–448. http://doi.org/10.1016/j.pbi.2008.05.005

Vargas, W. A., Mandawe, J. C., & Kenerley, C. M. (2009). Plant-Derived Sucrose Is a Key Element in the Symbiotic Association between Trichoderma virens and Maize Plants. *Plant Physiology*, *151*(2), 792–808. http://doi.org/10.1104/pp.109.141291

Vasconcelos, E. A. R., Santana, C. G., Godoy, C. V., Seixas, C. D. S., Silva, M. S., Moreira, L. R. S., … Grossi-De-Sa, M. F. (2011). A new chitinase-like xylanase inhibitor protein (XIP) from coffee

(Coffea arabica) affects Soybean Asian rust (Phakopsora pachyrhizi) spore germination. *BMC Biotechnology*, *11*, 14. http://doi.org/10.1186/1472-6750-11-14

Vitali, A., Pacini, L., Bordi, E., De Mori, P., Pucillo, L., Maras, B., … Giardina, B. (2006). Purification and characterization of an antifungal thaumatin-like protein from Cassia didymobotrya cell culture. *Plant Physiology and Biochemistry: PPB / Société Française de Physiologie Végétale*, *44*(10), 604–610. http://doi.org/10.1016/j.plaphy.2006.09.008

Viterbo, A., Harel, M., Horwitz, B. A., Chet, I., & Mukherjee, P. K. (2005). Trichoderma mitogen-activated protein kinase signaling is involved in induction of plant systemic resistance. *Applied and Environmental Microbiology*, *71*(10), 6241–6246. http://doi.org/10.1128/AEM.71.10.6241-6246.2005

Wahl, R., Wippel, K., Goos, S., Kämper, J., & Sauer, N. (2010). A Novel High-Affinity Sucrose Transporter Is Required for Virulence of the Plant Pathogen Ustilago maydis. *PLoS Biol*, *8*(2), e1000303. http://doi.org/10.1371/journal.pbio.1000303

Wallenfang, A. J. (2009). The WXXXE Effector Map Functions as a Potent and Specific Guanine Nucleotide Exchange Factor for CDC42. Retrieved from https://repositories.tdl.org/utswmed-ir/handle/2152.5/283

Wang, H., & Ng, T. B. (2002). Isolation of an antifungal thaumatin-like protein from kiwi fruits. *Phytochemistry*, *61*(1), 1–6.

Wang, K. L.-C., Li, H., & Ecker, J. R. (2002). Ethylene Biosynthesis and Signaling Networks. *The Plant Cell Online*, *14*(suppl 1), S131–S151. http://doi.org/10.1105/tpc.001768

Wang, K., Senthil-Kumar, M., Ryu, C.-M., Kang, L., & Mysore, K. S. (2012). Phytosterols Play a Key Role in Plant Innate Immunity against Bacterial Pathogens by Regulating Nutrient Efflux into the Apoplast1[C][W][OA]. *Plant Physiology*, *158*(4), 1789–1802. http://doi.org/10.1104/pp.111.189217

Wang, Z., Yan, S., Liu, C., Chen, F., & Wang, T. (2012). Proteomic analysis reveals an aflatoxin-triggered immune response in cotyledons of Arachis hypogaea infected with Aspergillus flavus. *Journal of Proteome Research*, *11*(5), 2739–2753. http://doi.org/10.1021/pr201105d

War, A. R., Paulraj, M. G., War, M. Y., & Ignacimuthu, S. (2011). Role of salicylic acid in induction of plant defense system in chickpea (Cicer arietinum L.). *Plant Signaling & Behavior*, *6*(11), 1787–1792. http://doi.org/10.4161/psb.6.11.17685

Weete, J. D., Abril, M., & Blackwell, M. (2010). Phylogenetic distribution of fungal sterols. *PloS One*, *5*(5), e10899. http://doi.org/10.1371/journal.pone.0010899

Wheeler, K. A., Lamb, H. K., & Hawkins, A. R. (1996). Control of metabolic flux through the quinate pathway in Aspergillus nidulans. *The Biochemical Journal*, *315 (Pt 1)*, 195–205.

Wilhite, S. E., Lumsden, R. D., & Straney, D. C. (2001). Peptide Synthetase Gene in Trichoderma virens. *Applied and Environmental Microbiology*, *67*(11), 5055–5062. http://doi.org/10.1128/AEM.67.11.5055-5062.2001

Wilson, T. J. G., Thomsen, K. K., Petersen, B. O., Duus, J. Ø., & Oliver, R. P. (2003). Detection of 3-hydroxykynurenine in a plant pathogenic fungus. *The Biochemical Journal*, *371*(Pt 3), 783–788. http://doi.org/10.1042/BJ20021797

Woo, S. L., Scala, F., Ruocco, M., & Lorito, M. (2006). The Molecular Biology of the Interactions Between Trichoderma spp., Phytopathogenic Fungi, and Plants. *Phytopathology*, *96*(2), 181–185. http://doi.org/10.1094/PHYTO-96-0181

Wösten, H. A. (2001). Hydrophobins: multipurpose proteins. *Annual Review of Microbiology*, *55*, 625–646. http://doi.org/10.1146/annurev.micro.55.1.625

Xu, L., Zhu, L., Tu, L., Liu, L., Yuan, D., Jin, L., … Zhang, X. (2011). Lignin metabolism has a central role in the resistance of cotton to the wilt fungus Verticillium dahliae as revealed by RNA-Seq-

dependent transcriptional analysis and histochemistry. *Journal of Experimental Botany*, *62*(15), 5607–5621. http://doi.org/10.1093/jxb/err245

Yadeta, K. A., & J. Thomma, B. P. H. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant Science*, *4*. http://doi.org/10.3389/fpls.2013.00097

Yang, D.-L., Yao, J., Mei, C.-S., Tong, X.-H., Zeng, L.-J., Li, Q., … He, S. Y. (2012). Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(19), E1192–1200. http://doi.org/10.1073/pnas.1201616109

Yedidia, null, Benhamou, null, & Chet, null. (1999). Induction of defense responses in cucumber plants (Cucumis sativus L.) By the biocontrol agent trichoderma harzianum. *Applied and Environmental Microbiology*, *65*(3), 1061–1070.

Yi, S. Y., Shirasu, K., Moon, J. S., Lee, S.-G., & Kwon, S.-Y. (2014). The Activated SA and JA Signaling Pathways Have an Influence on flg22-Triggered Oxidative Burst and Callose Deposition. *PLoS ONE*, *9*(2), e88951. http://doi.org/10.1371/journal.pone.0088951

Zdobnov, E. M., & Apweiler, R. (2001). InterProScan--an integration platform for the signature-recognition methods in InterPro. *Bioinformatics (Oxford, England)*, *17*(9), 847–848.

Zhang, S., & Xu, J.-R. (2014). Effectors and Effector Delivery in Magnaporthe oryzae. *PLoS Pathog*, *10*(1), e1003826. http://doi.org/10.1371/journal.ppat.1003826

Zhang, T., & Yao, Y.-F. (2015). Endophytic Fungal Communities Associated with Vascular Plants in the High Arctic Zone Are Highly Diverse and Host-Plant Specific. *PLoS ONE*, *10*(6), e0130051. http://doi.org/10.1371/journal.pone.0130051

Zhao, H., Xu, C., Lu, H.-L., Chen, X., St. Leger, R. J., & Fang, W. (2014). Host-to-Pathogen Gene Transfer Facilitated Infection of Insects by a Pathogenic Fungus. *PLoS Pathog*, *10*(4), e1004009. http://doi.org/10.1371/journal.ppat.1004009

Zhao, Z., Liu, H., Wang, C., & Xu, J.-R. (2013). Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics*, *14*(1), 1–15. http://doi.org/10.1186/1471-2164-14-274

Zuccaro, A., Lahrmann, U., Güldener, U., Langen, G., Pfiffi, S., Biedenkopf, D., … Kogel, K.-H. (2011). Endophytic Life Strategies Decoded by Genome and Transcriptome Analyses of the Mutualistic Root Symbiont Piriformospora indica. *PLoS Pathog*, *7*(10), e1002290. http://doi.org/10.1371/journal.ppat.1002290